Monitoring *Chrysaora hysoscella* (Cnidaria, Scyphozoa) in the Belgian part of the North Sea using eDNA

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Abstract

The use of Environmental DNA (eDNA) in monitoring ecosystems is now gaining attention in the field of research. The technique has shown a snapshot of the organisms present in the ecosystem being studied. Recent studies have demonstrated that the distribution and biomass of aquatic organisms can be estimated through detection and quantification of eDNA samples in the studied ecosystem. The approach is very rarely used in studying the detection and distribution of jellyfish in marine ecosystem. To investigate the technique's applicability in detecting and monitoring marine gelatinous zooplankton, eDNA was used to monitory the compass jellyfish (*Chrysaora hysoscella*) in the southern North Sea. eDNA samples were collected from the surface water of the nine studied stations in the Belgian part of the North Sea (BPNS) from 2014 to 2016. In this study, eDNA samples from October 2014, March, May, August and October 2015, January, March, May and August 2016 were extracted using CTAB. eDNA extracts were run in a qPCR for *C. hysoscella* eDNA detection and quantification.

This study detected *C. hysoscella* eDNA in the BPNS across the sampling months with a reduction in the frequency of detection in 2016. The target eDNA was found to be more common in Oostende then in Nieuwpoort and least in Zeebrugge stations. *C. hysoscella* eDNA was common and abundant in offshore stations and least in the shoreline stations. Peaks of eDNA abundance were recorded in spring, summer and autumn periods (October 2014, March, May and August 2016, March and August 2016). The recorded eDNA abundance was found to be not correlated with temperature ($p = 0.4254$). The results also revealed that the abundance of *C. hysoscella* eDNA somehow exhibited temporal and spatial variations. The results of this study imply that eDNA approach can be used to study the presence, patterns of distribution and the estimates of *C. hysoscella* biomass in the BPNS. This study confirms the broad potential of eDNA method in surveying ecosystems. The eDNA protocol used in the present study can be developed further to monitor jellyfish population in the BPNS obtaining a more detailed estimates of jellyfish abundance and distribution.

Chapter 1: General Introduction

1.1. Background of the study

Jellyfish blooms are considered natural population events observed in marine ecosystems (Hammer and Dawson, 2009) but the increasing incidence of jellyfish blooms has become a topic of recent scientific interest and research (Laakmann and Holst, 2013). In fact, several reports on huge jellyfish blooms have been documented in the Black Sea (Vinogradov et al., 1989), eastern Mediterranean Sea (Lotan et al., 1994), in the Bering Sea (Brodeur et al., 1999), off Japan (Uye and Ueta, 2004), northeast Atlantic and Mediterranean Sea (Licandro et al., 2010). These reports suggest that jellyfish blooms are not new events in marine ecosystem. According to Cho (2011), jellyfish blooms have been a problem since 1990s in the Sea of Japan, the East China and Yellow Seas, the Northern Benguela Current off Namibia, the Black Sea, the Baltic Sea, coastal Middle Eastern waters, and off the coasts of Spain and France. Recent evidence shows that there is a surge in the abundance of Cnidaria around the globe's oceans and blooms are now occurring more frequently in many water bodies (Purcell et al., 2007).

Reports of blooms of the most common jellyfish species in Belgian waters such as *Chrysaora hysoscella, Aurelia aurita* or *Cyanea lamarckii* have been circulated in the general and local media (Van Ginderdeuren et al., 2012). These species together with *Cyanea capillata* and *Rhizostoma octopus* are the most common jellyfish species in the North Sea with their blooms reported since 2000 (Van Ginderdeuren et al., 2012). At present, jellyfish research is attracting scientists around the globe because of their interactions with anthropogenic activities and the implications of their blooms on the society and on the economy (CIESM, 2001). One of the most important consequences of jellyfish blooms is its impacts and dangers on fisheries and aquaculture. Such blooms may lead to massive fish mortality (Doyle et al., 2008), damaged fishing equipment and vessels (Mills, 2001; Richardson et al., 2009 and Uye, 2008) or injured fishermen and reduced fish harvests (Dong et al., 2010; Quinoñes et al., 2012). Being considered as major planktonic predators (Barz and Hirche, 2005), increase in jellyfish population can also have negative effects on fish

larvae (Purcell et al., 2007; Boero, 2013). In addition, jellyfish stings may also threaten human population. Various reports worldwide show that jellyfish envenomation can cause injuries and deaths which subsequently results to losses in tourist revenue (Richardson et al., 2009). Additionally, when present in large numbers, jellyfish potentially cause clogging of salt water intakes of power plants leading to emergency situations for nuclear stations, significant economic losses, and threats to human lives (Purcell et al., 2007).

Recent advances on jellyfish proliferation studies link their blooms to several human-related factors like climate change, overfishing, eutrophication, industrialization and development of coastal regions (Lynam et al., 2004, 2005; Mills 2001, Purcell et al., 2007, Pauly et al., 2009). Even though there is an increasing research on jellyfish blooms worldwide in the last decade (Condon et al., 2012), there has been no single and clear explanation on the occurrence of such phenomena. Moreover, the ecological and evolutionary studies remain poorly integrated in studying jellyfish blooms (Lucas and Dawson, 2014). Lucas and Dawson (2014) stressed that research on jellyfish blooms is still far from elucidating which extrinsic (abiotic and biotic environmental) features of the modern seas and which intrinsic (functional biological) traits of these evolutionarily diverse taxa interact to cause jellyfish blooms.

The increase in jellyfish abundance has been widely argued and a general agreement on this issue has not been reached yet (Licandro et al., 2014). Some researchers are convinced that there is an increasing frequency of jellyfish blooms in the marine and estuarine ecosystems (Brodeur et al., 1999; Mills, 2001; Xian et al., 2005; Kawahara et al., 2006; Atrill et al., 2007; Licandro et al., 2010; Brotz et al., 2012) while others believed that the observed increase in jellyfish abundance is just a stage of up- and downward fluctuations characterizing jellyfish' long-term periodicity (Condon et al., 2013). Accompanying this issue and debate is the accepted fact that there is a lack of reliable jellyfish data (Purcell, 2009; Brotz et al., 2012; Condon et al., 2012). However, it is of great importance to evaluate jellyfish population sizes quantitatively to monitor population changes over time regardless of the cause of jellyfish outbreaks (Brierley et al., 2004).

1.2. Statement of the problem

Since jellyfish blooms are becoming more frequent, an urgent need to enhance our understanding on their population dynamics and distribution is necessary (Ionescu et al., 2016). Since blooms of *C. hysoscella* in the Belgian part of the North Sea (BPNS) and its negative consequences in tourism have been reported in the Flemish media (Van Ginderdeuren et al., 2012), monitoring this jellyfish species is important to manage its future increase in number. According to Uye et al. (2003), to properly evaluate and quantify the real impact of jellyfish in the marine ecosystem, it is necessary to assess their temporal and spatial distribution.

In addition, conventional methods of studying jellyfish distribution and abundance in the marine environment have several limitations (Fearon et al., 1991; Brierley et al., 2001; Brierley et al., 2004). Moreover, the intrinsic characteristics of jellyfish species like their large size, fragility and non-dispersed distributions contribute to the problems on field sampling and laboratory experiments involving many gelatinous zooplankton species (Raskoff, 2003). These limitations and problems necessitate the development of a more effective technique that would provide quantitative estimates of jellyfish abundance. Hence, the emerging environmental DNA (eDNA) approach for monitoring marine species can be a candidate to fill the loopholes brought about by the conventional methods for surveying jellyfish.

Specifically, the present study aims to answer the following questions:

1. Does the designed *C. hysoscella* primer used in the present study specifically detect the target DNA fragments from the collected water samples?
2. Can the presence of the jellyfish *C. hysoscella* be determined by using its eDNA in the water samples collected from the surface of the nine study locations in the BPNS?
3. Can the spatial and temporal distribution variations and the abundance of the jellyfish *C. hysoscella* be evaluated based from the qPCR analysis of its eDNA in the water samples collected from the surface of the nine study locations in the BPNS?

4. Is the eDNA approach applicable and effective in monitoring the presence, distribution and abundance of a jellyfish species from marine environment?

1.3. Significance of the study

To know if jellyfish blooms are happening more frequently and if such outbreaks are lasting longer compared to the previously recorded blooms, long time-series observations are necessary (Kogovšek et al., 2010; Licandro et al., 2010; Uye, 2008). This is not available in the literature for *C. hysoscella* in the BPNS. Monitoring the spatial and temporal distribution as well as the abundance of *C. hysoscella* will improve our understanding of their population dynamics and bloom frequency. This study could provide a baseline for a long-term monitoring study concerning the distribution and abundance of *C. hysoscella* and of other jellyfish species in the BPNS. Moreover, at present, there are only limited studies published on literature that are linked to jellyfish in the North Sea (Lynam, 2004; Lynam et al., 2005; Van Ginderdeuren et al., 2012; Laakmann and Holst, 2014; Vansteenbrugge et al., 2015). Thus, the present study could also provide additional scientific knowledge to the scarce collection of jellyfish research in the North Sea. The present study would also evaluate the applicability and effectivity of using eDNA as a tool for monitoring jellyfish distribution and abundance. Thus, this study contributes to the application of eDNA in monitoring marine environments and organisms.

Chapter 2: Literature Review

2.1. Jellyfish diversity

The term “jellyfish” is used and limited only for a defined plankton functional group – the gelatinous carnivores under Phyla Cnidaria and Ctenophora (Licandro et al., 2014). Phylum Cnidaria to which jellyfish belongs contains approximately 10,211 species around the globe (Appletans et al., 2012). According to Jankowski et al. (2008), this diverse phylum is composed of anemones, corals, medusae and other polyps and is unusually prosperous in the marine environment (7000+ identified or described species). Phylum Cnidaria is composed of three main groups namely: Scyphozoa, Cubozoa and Hydrozoa. As stated by Rizman-Idid et al. (2016), the World Register of Marine Species recognized 187 Scyphozoan species and 46 Cubozoan species. Four orders of the Scyphozoan jellyfish are widely distributed: Coronatae (crown jellyfish), Rhizostomeae (true jellyfish), Stauromedusae (stalked jellyfish) and Semaestomeae (sea nettle) (Kramp, 1961; Pitt, 2000; Brusca and Brusca, 2002; Shao et al., 2006 and Richardson et al., 2009). The box jellyfish or the Cubozoan jellyfish are distributed into two orders, the Carybdeida and Chirodropida (Gershwin 2005a, 2005b, 2006a, 2006b & Daly et al., 2007). The most diverse group of the Cnidarian jellyfish is the Hydrozoan group with 3,676 recognized species in the World Hydrozoa database (Schuchert, 2015). Cnidarian jellyfish differ in size from few millimeters to a few meters and can be described as solitary like the medusae species of Hydrozoa, species of Scyphozoa and Cubozoa or colonial as the Hydrozoan siphonophores (Pugh, 1975; Gamulin and Krsinie, 1993).

2.2. Study species: *Chrysaora hysoscella* (Linnaeus, 1767)

Commonly known as “compass jellyfish”, *C. hysoscella* (see Figure 1) is a yellowish medusa characterized by 16 gold-brown or yellow-brown lines or bands (the V-shaped markings) on the upper surface of the umbrella radiating from the central region. The bell is hemispherical and flattened with 32 pigmented semi-circular marginal lappets and 24 tentacles. This species has four very long oral arms with several fringings in the upper part

and are scallop-edged in the lower part. Compared with other scyphozoan jellyfish species, *C. hysoscella* is a protandric hermaphrodite species (Vaissiere, 1984; Jellyrisk, 2014; Cornelius, 2004).

Generally, this jellyfish species appears in cold and temperate waters and can live in waters of temperature 4°C to 28°C (Vaissiere, 1984). It also occurs in the upwelling areas such as in the Benguela ecosystem extending from northern Namibia to south of Cape Point in South Africa (Sparks et al., 2001). Several *C. hysoscella* outbreaks were recorded in different parts of the globe. It was found abundant in the Southern Adriatic Sea and in the Northern Ionian Sea (Scalera-Liaci, 1991). Although it was scarcely found in the Northern Adriatic Sea (Zavodnik, 1991), early appearance of the species was already reported (Issel, 1922). Coastal blooms of this species were also recorded in the spring of 1989. It was observed in the Gulf of Trieste (Del Negro et al., 1992). *C. hysoscella* sightings were accounted for around 3.5% of total jellyfish appearance along the Ligurian Riviera (Carli, 1991). The species was also reported to appear in large number in Belgian coast (Van Ginderdeuren et al., 2012). To date, there are no recent studies dealing with the patterns of distribution and abundance of *C. hysoscella* over time.

Figure 1. *C. hysoscella* in its environment. Source: Jellyrisk, 2014

Knowledge on the toxicity of *C. hysoscella* seems to be very scarce (Del Negro et al., 1991, 1992; Kokelj et al., 1990; Parodi et al., 2009). The earliest documentation of its toxicity was a report on cutaneous lesions caused by this jellyfish in subtropical waters (Vine, 1986). Despite of this limited knowledge on its envenomation, the species is considered a dangerous one due to its wide wounding surface, long tentacles and a large umbrella (Vaissiere, 1984). According to Dr. Tom Doyle of the Coastal & Marine Resource Center (CMRC) of the University College Cork, Ireland, the species has the ability to sting and the

sting may pose high risk to human health (Jellyrisk, 2013). Dermatitis accompanied by itching and burning sensation due to contact with *C. hysoscella* were observed within 20 minutes after contact. These effects disappeared within few hours (Kokelj et al., 1989). Massive stinging events took place in the zone of Grado (Gulf of Trieste, Italy) between May–August 1997 injuring a total of 90 people. Sampling during these periods revealed that *C. hysoscella* was the only dermatoxic jellyfish constantly appearing during that time (Kokelj et al., 1999). Studies dealing with fractions of this species tested for dermatoxicity through scratch-patch test in some volunteers showed that five out of 25 volunteers reacted to the jellyfish and demonstrated itching, erythema and edema after 48 hours (Del Negro et al., 1991) while in some studies volunteers showed itching and burning sensation within 40 seconds after the contact and developed erythema and vesicles after three minutes (Del Negro et al., 1992). The hemolytic fraction of the venom coming from *C. hysoscella* showed at least a partial proteinaceous nature with the presence of a cationic protein (Del Negro et al., 1991). It is possible to state from Del Negro et al. (1991) that *C. hysoscella* nematocysts have a cytotoxic and dermatoxic activity, even though the substance (or the substances) involved are not known at present. Recent studies showed that *C. hysoscella* crude extract induced 55% mortality with 0.14 µg/µL IC50 in cultured keratinocytes at the dose of 0.15 mg/mL proteins when assessed with neutral red assay (Parodi et al., 2009). When toxicity is concerned, *C. hysoscella* is not harmless and the absence of previous reports on its toxicity might be due to its sporadic presence. Therefore, accumulations during the holiday season might involve a risk to public health (Del Negro et al., 1991, 1992).

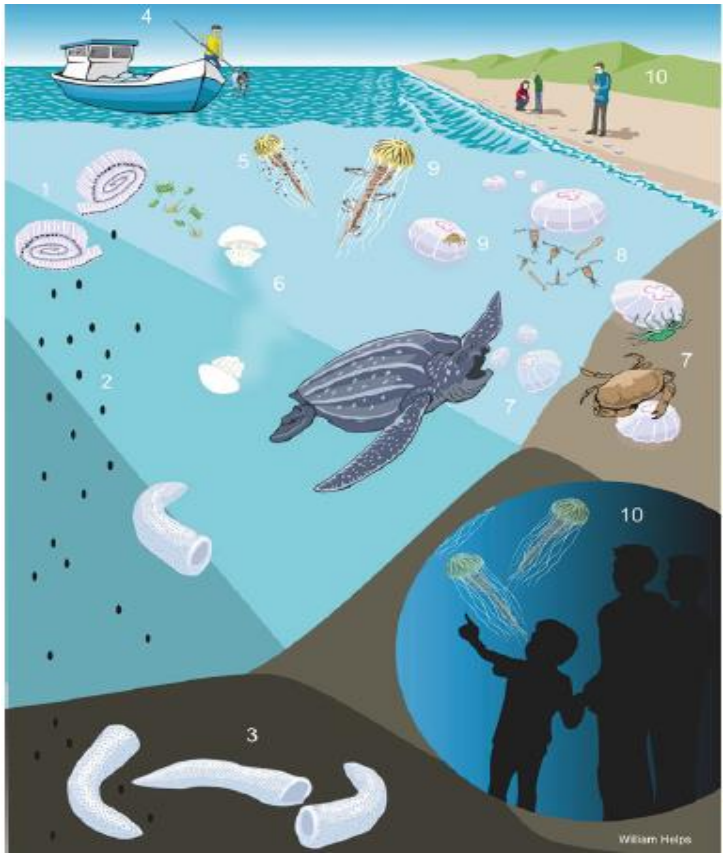
2.3. Ecological and societal roles of jellyfish

Although various reports had been established on the negative impacts of jellyfish (especially when blooms occur), their presence whether in small or large number brings some ecological and societal benefits. Generally, gelatinous marine species contributes to four categories of ecosystem services defined by the Millennium Ecosystem Assessment (Doyle et al., 2014). These ecosystem services are regulating, supporting, provisioning and cultural services (see Figure 2). Most of the Cnidarian jellyfish are often considered as

stressors of the marine ecosystems or can be indicators of highly disturbed systems (Doyle et al., 2014) but they can also be described as ecosystem service providers.

Figure 2. The ecosystem services provided by the jellyfish.

Regulating services: (*1*) salps consume phytoplankton and transport carbon to the benthos via faecal pellets (*2*). (*3*) Accumulation of jellyfish carcasses (pyrosomes) on the seabed plays an important role in the transfer of carbon from surface waters to the benthos. Provisioning services: (*4*) jellyfish harvested for food and other uses. Supporting services: (*5*) sloppy feeding provide nutrients to support primary production, (*6*) swimming jellyfish contribute to oceanic mixing due to displacement of water as they move through it, (*7*) jellyfish provide a prey source for hundreds of different animals, (*8*) jellyfish are important predators in pelagic marine systems, and (*9*) jellyfish provide habitats and refugia for a large variety of taxa.



Source: Scientific illustration by William Helps as adopted from Doyle et al. (2014).

2.3.1. Regulating services

In this regard, jellyfish contributes to carbon sequestration which in effect helps to regulate climate as they become sources and sinks for greenhouse gases. Lebrato et al. (2012) indicated that accumulation of jellyfish remains at the seabed (known as jellyfish-falls) also plays a significant role in the movement of carbon from the surface waters to the seabed. In fact, a single jellyfish-fall may deposit carbon which is four times the annual carbon input to the seabed (Lebrato and Jones, 2009) with this, it is possible that accumulation of jellyfish remnants in the seabed can alleviate some of the losses of carbon from the soon to be decreasing classical phytoplanktonic carbon flux (Lebrato et al., 2012).

Additionally, jellyfish species can also contribute to pest regulation. Kideys (2002) reported that the introduction of of *Mnemiopsis* sp. (through ballast water) into the Black Sea in 1980s had disastrous consequences for the pelagic fish populations which led to large economic losses to the Turkish fishing industry. The introduction of an invasive jellyfish *Beroe sp*. in 1997, a predator of *Mnemiopsis*, has helped the ecosystem to recover. The invasive jellyfish consequently disappeared with the decline of its prey showing that the introduction of this invasive species did not pose any threat to the ecosystem (Kideys, 2002).

Lastly, jellyfish can also play a role in biodiversity regulation which can impact the delivery of ecosystem services (Díaz et al., 2005). For example, when jellyfish are at low densities they may serve as keystone species (Piraino et al., 2002; Pauly et al., 2009) and act as the main predator of commercially important or numerically abundant fish populations (Purcell, 1989; Purcell and Grover, 1990). By controlling such fish populations (by preying on fish eggs and larvae), jellyfish indirectly frees resources for less well-established fish species leading to enhanced local biodiversity (Boero et al., 2008).

2.3.2. Provisioning services

Ecosystem resources that deliver food, fiber and fuel are considered to provide provisioning services (Anonymous, 2005). Jellyfish are considered as traditional food in many Asian countries. In China for example, it is a tradition to prepare jellyfish salad during a wedding or formal banquet while in Japan jellyfish are served as an appetizer (Hsieh et al., 2001; Omori and Nakano, 2001). As the population in China is increasing rapidly, the demand for jellyfish as food also surges (Doyle et al., 2014). There are at least 10 jellyfish species (all are Rhizostomeae) which are harvested commercially mainly from China, Japan and other parts of South East Asia (e.g. Indonesia, Malaysia, the Philippines, Thailand, Singapore), with *Rhopilema esculentum* as the most important one (Doyle et al., 2014). The consumption of jellyfish is believed to bring health benefits even though they have

a low nutritional value (Doyle et al., 2007; You et al., 2007; Hsieh et al., 2001; Addad et al., 2011).

Jellyfish can also provide novel biomolecules. Indeed, one of the paramount benefits that jellyfish have provided to society was the discovery and subsequent development of the green fluorescent protein (GFP) (Tsien, 1998; Chalfie and Kain, 2006; Zimmer, 2009). Since the discovery of fluorescent proteins (FP) in jellyfish and other marine fauna, science was able to produce different variety of such FP ranging from violet to far red (Chudakov et al., 2010). FPs and their derivatives have shown promising and wide applications in research and biomedical fields (Chudakov et al., 2010; Doyle et al., 2014). Extensive research efforts are made to extract other biomolecules from jellyfish species. Recently, 'qniumucin', a mucin molecule was found in six jellyfish specimens examined by Ohta et al. (2009). This molecule is a glycoprotein with lubrication and protective functions. This mucin molecule has important potential as currently, there are no methods to produce large quantities of mucins artificially for therapeutic use (Ohta et al., 2009) whereas jellyfish could be harvested in sufficient quantities to meet this demand (Doyle et al., 2014).

2.3.3. Supporting services

2.3.3.1. Jellyfish as prey

Jellyfish species may also serve as prey (therefore food) to other animals (Pauley et al. (2009), Purcell (1997), Ates (1988) and Arai (1988, 2005)). Various records show that some other jellyfish taxa, fish, arthropods, molluscs, reptiles and birds regularly or occasionally prey on gelatinous organisms. For example, Purcell (1991) showed that *Aurelia aurita* was a prey to *Cyanea capillata* (a scyphomedusae). *Aequorea victoria* (a hydromedusae) was proven to feed up to 10 jellyfish species which are mostly hydromedusae species (Purcell, 1991). One of the few cases where a jellyfish feeds solely on another jellyfish is *Beroe cucumis* (a ctenophore) and *Mnemiopsis* feeding greatly on *Bolinopsis infundibulum* (a ctenophore) (Doyle et al., 2014). Fish are one of the most common predators of jellyfish. Various reports on different fish species feeding on jellyfish have been

documented and compiled by Arai (1988, 2005). Fish preying on jellyfish so far includes 69 fish species in 34 families and this number tends to increase with time (Arai, 2005). In fact, Pauly et al. (2009) revealed a total of 124 fish species which were reported to consume sporadically or regularly on jellyfish. This number was obtained after using information from FishBase, data published by Arai (1988, 2005) and other relevant resources.

2.3.3.2. Jellyfish as predators

The jellyfish group is certainly considered as a very important predator in the pelagic marine environment (Pauly et al., 2009). Because of their prolonged existence and evolutionary head start on other taxa (evolved 500 to 540 MYA) (Richardson et al., 2009) and their different array of armature and prey-capture mechanisms, jellyfish have perhaps shaped pelagic marine ecosystems (Doyle et al., 2014). Furthermore, Doyle et al. (2014) emphasized that due to jellyfish' diverse way of feeding behaviors and varying body sizes, they become capable of feeding on preys of different sizes and types ranging from micro-heterotrophs, zooplankton, other jellyfish and fish. Several factors contribute to the different diets observed in jellyfish species – different feeding mechanisms, prey behavior and escape ability, nematocyst and colloblast structures, toxicity of nematocysts, life cycle and life history (Purcell, 1997; Boero et al., 2008). Most species of scyphomedusae, hydromedusae and siphonophores are carnivorous and have wide-ranging diets which includes a variety of zooplankton taxa from copepods, veliger larvae, fish eggs/larvae and other jellyfish but some are considered specialists as they feed on specific prey like *Hippopodius hippopus* (a siphonophore) preying only on ostracods (Purcell, 1981). As predators, jellyfish have the tendency to consume and deplete resources that are vital and available to commercial fish stocks (Brodeur et al., 2002; Lynam et al., 2005; Hong et al., 2008) and this ability brings a serious problem to the fish industry. When jellyfish in the marine ecosystem occurs in large numbers (during blooms) their group prey-consumption rate can be so high

that their prey-consumption impacts directly or indirectly the population size of other zooplankton organisms including larval fish (Purcell, 1989).

2.3.3.3. Jellyfish as habitats and nurseries

Considering the large size of most jellyfish as compared to other planktonic organisms, their slow swimming characteristics compared with nektonic animals of a similar size or mass, their complex morphology combined with a marine environment that is unusually barren of physical habitat, jellyfish create structurally intricate assortment of surfaces and constantly changing place or habitat for other marine organisms. Jellyfish provide a three-dimensional structure in pelagic habitats like coral reefs and oyster beds do (Breitburg et al., 2010). Three possible relationships between jellyfish and symbionts can be described. These relationships are based on how symbionts use and exploit this largely transparent but solid substrate (jellyfish). Jellyfish can provide (1) pelagic refugia or shelter, (2) pelagic substratum and (3) a host for algal symbiotic associations (Doyle et al., 2014).

Pelagic refugia: The biological relationship between the jellyfish and juvenile fish is considered as the best example of the jellyfish' role in providing shelter for marine organisms. This interaction is commonly considered to be a facultative symbiotic relationship. It occurs only when a jellyfish becomes the only available refuge in a pelagic environment for juvenile fish (Doyle et al., 2014). This claim has been supported by Castro et al. (2001) as they showed that there are more than 333 fish families that display aggregative behavior beneath floating objects. From these, nine fish families are known to interact with jellyfish. According to Mansueti (1963), four of these families are pelagic, three are demersal and two are deep-sea inhabitants. The juvenile fish interacting with the jellyfish may obtain food from the association by feeding directly on (a) prey encountered by the fish as the jellyfish swims through the water column, (b) zooplankton taken from the host, (c) the jellyfish itself and (d) predation upon amphipod parasites present on the host jellyfish (Mansueti, 1963; Purcell and

Arai, 2001). Jellyfish' ability to provide shelter and food to fish juveniles tends to increase the survival of such juveniles to adulthood (Brodeur 1998; Lynam and Brierley, 2007). Doyle et al. (2014) claimed that the interaction between the juvenile fish and the jellyfish does not exclude the competition or predation for resources between the two. Instead, the relationships are complex and not always detrimental to the fish.

A pelagic substratum: In addition to their shelter provision to juvenile fish, jellyfish also provide habitat for a wide range of taxa starting from microorganism to invertebrates including crustaceans like crabs, shrimp, brachyuran, barnacles, copepods, amphipod (Perissinotto and Pakhomov, 1997; Pagès, 2000), digeneans (Martorelli, 2001), pycnogonids (Pagès et al., 2007), and protists (Moss et al., 2001). A study revealed that an amphipod ectosymbiont *Hyperia medusarum* parasitizes the jellyfish *Phacellophora camtschatica* through direct consumption of the host's tentacles and other tissues, (with 100% infestation rates at times and as high as 446 individuals parasitizing a single jellyfish (Towanda and Thuesen, 2006). The same study demonstrated the symbiotic interaction between brachyuran crabs and jellyfish. At least eight species of brachyuran crabs are found to interact with jellyfish (Towanda and Thuesen, 2006). Martorelli (2001) showed that other parasitic marine organisms use jellyfish as their intermediate hosts before infecting their definitive host (normally a fish).

Hosts for algal symbiotic associations: Many jellyfish groups are in symbiotic relationship with the photosynthetic dinoflagellates zooxanthellae. For example, the golden jellyfish (*Mastigias papua*) exhibits daily horizontal migrations and has a behavior of avoiding shadows in landlocked marine lakes in Palau in to keep their zooxanthellae in direct sunlight (Dawson and Hamner, 2003). Another unusual behavior of a jellyfish harboring zooxanthellae is that of the upside-down jellyfish *Cassiopea sp*. The striking behavior of this jellyfish is that they spend most of their time upside down resting on the seabed to make

the most of photosynthesis as they contain zooxanthellae in their oral arms. Their activities have shown to increase the benthic oxygen production almost 100-fold (Welsh et al., 2009). Additionally, the algal host pleustonic jellyfish *Velella velella* is known to be found in surface waters across the globe in tropical and temperate open ocean waters (Purcell et al., 2012). The zooxanthellae on these jellyfish perform photosynthesis and support the host's energy budget (Doyle et al., 2014).

2.3.4. Cultural services

Jellyfish species are now being displayed in large aquaria fascinating people with their unfamiliar forms such as having floating parachutes with trailing tentacles (Hardy, 1956). To this purpose, the Monterey Bay Aquarium in California has been known to show exquisite displays of jellyfish such as the Pacific Sea Nettle (*Chrysaora fuscescens*). From 2002 to 2008, the aquarium showed a special exhibition (Jellies: Living Art) that showcased 25 species of jellyfish with works of art portraying jellyfish (Monterey Bay Aquarium, 2004).

Humans have also exploited the ecosystems sheltering jellyfish populations as ecotourist attractions as shown by the well-known Jellyfish Lake (previously Ongeim'l Tketau) in Palau, a landlocked marine lake on Eil Malk Island. The lake houses several million-golden jellyfish (*Mastigias sp*.) and common jellyfish (*Aurelia sp*.) yearly (Dawson et al., 2001).

2.4. Anthropogenic causes of jellyfish bloom

The ability of the jellyfish to bloom lies to their capacity to reproduce both sexually and asexually (Purcell et al., 2007). Their life cycle includes a planktonic stage or a benthic polyp stage (Boero et al., 2007) (see Figure 3). According to Lucas (2001), most coastal jellyfish reproduce asexually through budding from an attached stage in the life cycle, a scyphistoma for scyphozoans, and a hydroid (often colonial) for hydromedusae. These attached stages were referred to as polyps (Purcell et al., 2007). These polyps have the tendency to produce

more polyps by budding and many jellyfish can be budded from a single polyp. Meanwhile, cubozoan polyps are an exemption since they develop into individual jellyfish without budding. Swimming jellyfish in turn reproduce sexually. They have excessive fecundity and may brood the larvae, which settle to become polyps. Temperate and tropical jellyfish life cycle could differ. According to Lucas (2001) in the tropics, jellyfish production can occur all year round while temperate species usually have an annual cycle. The small jellyfish, about 1 to 2 mm ephyrae for scyphozoans are being produced in fall or spring, and they grow and become sexually mature over the summer. By contrast, ctenophores and siphonophores (also cnidarians) are holoplanktonic and lack an attached stage in the life cycle. The siphonophores also have asexual stage of reproduction followed by sexual reproduction. Ctenophores are considered hermaphroditic and characterized by having direct development and high fecundity. Because of these modes of reproduction, siphonophores and ctenophores occur in multiple generation in a year, in contrast to many species in the other jellyfish taxa (Purcell et al., 2007). Many hydrozoans are well-known for reproductive diversity (Boero et al., 2002). Many hydromedusae are holoplanktonic while this is uncommon in scyphozoans, some hydromedusan jellyfish reproduce asexually (Boero et al., 2002). Therefore, the capacity of ctenophores and pelagic cnidarians to bloom in good conditions is inherent.

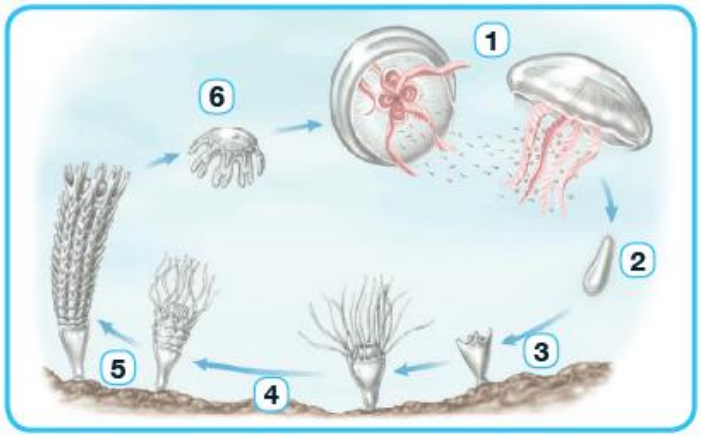


Figure 3. General life cycle of jellyfish.
(1) Adults reproduce sexually with external fertilization; (2) The planula larvae swims freely for a short time; (3) The planula fix on a substrate and transform into a polyp; (4) The polyp undergoes the strobilation process; (5) Dividing itself in segments and producing ephyras; (6) Each ephyra or juvenile jellyfish swims freely until it becomes a new adult jellyfish. *Source: Jellyrisk, 2014*

Naturally, jellyfish may reach great densities in enclosed embayment and at physical oceanographic discontinuities such as fronts (Graham et al., 2001). Some of these population fluxes are strongly seasonal in nature, reflecting the life cycle characteristics of

the Cnidarian species involved while the peaks in abundance are reflected by seasonal variations to the structure of marine plankton communities (Feigenbaum and Kelly, 1984 as cited by Purcell et al., 2007). Yet, studies showed that blooms of some species are happening more frequently and for longer extent in recent years, and this observation can be a natural response to the altered functioning of marine ecosystems (Mills, 2001; Purcell et al., 2007; Richardson et al., 2009). The recently observed fluctuations in jellyfish abundance have hypothetically associated with climate indices such as the North Pacific Decadal Oscillation (NPDO) and the North Atlantic Oscillation (NAO) (Lynam et al., 2004, 2005b; Purcell, 2005) as well as with the variation in sea surface temperature (SST) (Lynam et al., 2011), salinity (Bastian et al., 2011a), pH (Attrill et al., 2007), eutrophication (Purcell et al., 2007) and habitat modification (Richardson et al., 2009). Climate change could possibly alter marine ecosystem dynamics which could influence the distribution and abundance of marine planktonic communities (Hays et al., 2005). Modification of oceanographic dynamics may also be linked to increases in abundance related to opportunistic expansion, following decreased predatory pressure as a result of declining fish abundance due to commercial fisheries (Pauly et al., 1998; Mills, 2001; Lynam et al., 2006). Changes occurring in many of the coastal waters worldwide as consequences of anthropogenic activities and practices could benefit pelagic cnidarian and ctenophore populations over fish (Purcell et al., 2007). Several authors have speculated how some of these factors may contribute to jellyfish blooms (Arai, 2001; Mills, 2001; Purcell et al., 2001b; Parsons and Lalli, 2002; Oguz 2005a, b; Purcell, 2005; Graham and Bayha, 2007). The previously mentioned factors possibly causing jellyfish blooms are linked to human activities and these factors might act synergistically (Purcell et al., 2007; Richardson et al., 2009).

2.4.1. Climate change

Long-term data revealed fluctuations of pelagic cnidarian and ctenophore abundance that are correlated with temperature (Purcell, 2005). As reviewed by Purcell et al. (2007), the most studied moderate-temperature jellyfish species (18 of 24) have been reported to increase in abundance in warm temperatures. The NAO

Index, have been related to jellyfish abundances instead of, or in addition to, environmental measurements (Lynam et al., 2004, 2005; Molinero et al., 2005). Increased temperature may result to jellyfish proliferation (Purcell et al., 2007; Boero et al., 2002) and may affect and shift their population distributions (Sullivan et al., 2001; Faase and Bayha, 2006; Hansson, 2006). Rising temperature also changes the timing and duration of the pelagic stages of jellyfish reproduction (Purcell et al., 2007). Raskoff (2001) noted that one meroplanktonic hydromedusan species increased during an El Niño, contrastingly, a holoplanktonic species did not. *Chrysaora melanaster* was found to increase in the Bering Sea with warming from the shift to a positive NPDO from 1976 to 1977, increasing most dramatically in the 1990s, but then decreased with further warming since 2000 (Brodeur et al. in press). However, the trends in jellyfish and ctenophore abundance in warm conditions were found to be contradictory to the trends in the North Sea. Lynam et al. (2004) showed that the jellyfish *Cyanea capillata, Cyanea lamarckii* and *Aurelia aurita* increased in abundance with decreased temperature in the North Sea during 1971 to 1986. Attrill et al. (2007) conducted a different study using Continuous Plankton Recorder (CPR) data on nematocyst occurrence as an index of jellyfish abundance in the North Sea during 1958 to 2000. The study indicated that nematocysts were increased with increasing NAO Index, but not with temperature. Both studies discussed the fact that the trends in jellyfish abundance are dependent on the region of the North Sea and on the current regime. As climate warming carries on, the NAO is predicted to move into a stronger positive phase (Osborn, 2004), which according to Attrill et al. (2007) would lead to a greater abundance of jellyfish. Further related investigations of jellyfish in the North Sea seem necessary to resolve these apparently conflicting results.

2.4.2. Cultural eutrophication

Cultural eutrophication is eutrophication that is accelerated by human activities. Eutrophication is a phenomenon linked with increased nutrients, changed nutrient ratios and increased turbidity in coastal regions where humans develop

(Daskalov, 2002). Indeed, this phenomenon is considered to be one of the major global pollution problems (Howarth, 2002). In effect, increased nutrients during eutrophication lead to greater biomass at all trophic levels (Daskalov, 2002). The availability of more foods for the polyps and jellyfish escalates asexual production of jellyfish (Purcell et al., 1999a; Stibor and Tokle, 2003) and their sexual reproduction (Lucas, 2001).

The recent jellyfish blooms in the Yangtze River estuary was thought to be related to the increased nutrient input in the river (Zhang et al., 1999) as a result of rampant use of nitrogen fertilizer after the 1970s (Glibert et al., 2005). Since 1993, annual blooms of *Rhizostoma pulmo* and *Cotylorhiza tuberculate* (both rhizostome scyphomedusae) in Mar Menor, Spain was attributed to agriculture and development which elevated nitrate levels 10-fold while wastewater treatment decreased phosphate by one-tenth. Both conditions facilitated the observed blooms (Pérez-Ruzafa et al., 2002). These cases clearly suggest that high nitrogen ratios may favor jellyfish blooms (Purcell et al., 2007). Eutrophication can also induce intricate changes in the food web favoring the increase in jellyfish abundance (Greve and Parsons, 1977; Nagai, 2003; Uye, 1994). As a result of eutrophication, dissolve oxygen level in bottom waters is usually reduced (hypoxic condition) (Breitburg et al., 2003). Reports indicated that fish may avoid or die in waters with ≤2–3 mg O_2 per liter (Breitburg et al., 2001). However, many jellyfish species are able to tolerate waters with ≤1 mg O_2 per liter (Purcell et al., 2001b). Supporting this claim were studies on ctenophores and *Aurelia labiate* which showed great tolerance to low dissolved oxygen (Rutherford and Thuesen, 2005; Thuesen et al., 2005). Condon et al. (2001) showed that polyps are also tolerant in hypoxic conditions and may find additional home where other epifauna is reduced in hypoxic waters (Ishii, 2006). Planktonic ctenophores and some other jellyfish species don't have a polyp stage, and these species may persist where hypoxic bottom waters prevent others with vulnerable benthic stages (Arai, 2001). Eutrophication decrease water clarity and light penetration, which may alter the feeding environment benefiting gelatinous

predators over fish. Fish are considered feeders whereas jellyfish are not. In effect, turbid water could reduce feeding by fish but not in jellyfish (Purcell et el., 2007).

2.4.3. Fishing

Pelagic cnidarian and ctenophores benefit positively from fishing activities by eradicating the predators of these gelatinous species (Purcell and Arai, 2001; Arai, 2005). Various reports have shown that gelatinous organisms are consumed by commercially important fish species like *Peprilus triacanthus*, *Onorhynchus keta* and *Squalus acanthias* (Arai, 1988, 2005; Purcell and Arai, 2001). Other predators of gelatinous species were found to decline partly due to fishing (Purcell el at., 2007). For example, *Dermochelys coriacea* (leatherback turtle) populations have dwindled intensely in the Pacific Ocean according to Spotila et al. (2000). Zooplanktivorous forage fish species are known to feed on jellyfish and other gelatinous species. Forage fish species competes with the other gelatinous predators for food. The diets of these two groups of predators overlap (both feeding on gelatinous organisms). Therefore, fishing for zooplanktivorous forage fish species may have two effects: it removes potential competitors for jellyfish feeding (beneficial for other predators) and may lessen the number of predators that feed on the gelatinous organisms (beneficial for the prey) (Purcell and Sturdevant, 2001; Brodeur et al., unpubl.).

Mills (2001) put forward that the observed rise in the siphonophore *Nanomia cara* in the Gulf of Maine could be a consequence of the reduction of zooplanktivorous fish through fishing. The decrease in the number of zooplanktivorous fish populations was evidenced by the replacement of some fish in the Black Sea and the Benguela Current by ctenophore and jellyfish (Shiganova, 1998; Daskalov, 2002; Oguz, 2005b; Lynam et al., 2006). Overfishing other organisms like mollusks and crustaceans may also result in severe changes in the ecosystem (Jackson et al., 2001) which may facilitate jellyfish and ctenophore blooms. Pauly and Watson (2003) considered overfishing or overharvesting of marine resources as a severe problem. Several discussions on how overharvesting one resource after

another, in combination with other ecosystem damage, may result to greater jellyfish and ctenophore populations (Jackson et al., 2001; Daskalov et al., 2007).

2.4.4. Aquaculture

Purcell et al. (2007) pointed out that aquaculture brings unintended benefits to jellyfish populations in different ways. Firstly, using feeds in aquaculture may result to eutrophic waters and results to consequences that were previously mentioned in this paper, most of which end up in increasing jellyfish and ctenophore populations. Secondly, the used aquaculture structures may serve as additional substrates on which benthic stages of jellyfish life cycle may live and attach leading to a more successful maturation, development and greater jellyfish production. Thirdly, the harvesting of forage fish (such as menhaden, anchovies and sardines) as aquaculture feed unintentionally benefits the jellyfish and other jellyfish predators (Kristofersson and Anderson, 2006). The removal of such zooplanktivorous fish may provide opportunities for gelatinous population to grow as previously mentioned. Aquaculture may also enhance jellyfish population intentionally. This has been observed in China where *Rhopilema esculentum* is being cultured in response to a dramatic decline in catches of such species. About one hundred million 1 cm jellyfish are released annually in the marine environment in February and March, and by August, these released jellyfish are expected to 50 cm in length from which they can be caught for use. The success of jellyfish aquaculture in China had triggered Malaysia to start a similar aquaculture program in 2004 (Anonymous, 2004). Purcell et al. (2007) asserted that such jellyfish enhancement programs seem certain to have ecological consequences that are unstudied.

2.4.5. Construction

Construction pertains to different human destructions to aquatic habitats which added structures to or had modified the characteristics of the coastal waters. Such structures may include marinas, oil platforms, docks, breakwaters, and artificial reefs. These structures provide surfaces for attachment of polyps although

the importance in not known (Purcell et al., 2007). Majority of the recorded jellyfish blooms took place in heavily populated areas surrounding semi-enclosed water bodies. These areas are often characterized by eutrophication, fishing and extensive construction (Arai, 2001). Hydrological alterations may affect the Yellow Sea, where *Nemopilema nomurai* blooms may originate (Purcell et al., 2007). Alterations in the water flow towards the Black Sea were seen to contribute changes that benefits jellyfish and ctenophores (Oguz, 2005b). Establishment and activities of nuclear and thermal power plants near coastal regions also contribute to jellyfish bloom. These installations use coastal waters for cooling and discharge the heated water back into the marine environment (Purcell et al., 2007).

2.4.6. Alien invasion

Accidental introduction of jellyfish species in many places worldwide were documented (Dawson et., 2005) and had caused serious destruction of the ecosystem and economic losses (Graham and Bayha, 2007). Jellyfish can be transported from one place to another mostly via ballast water and sometimes aquarium trade (Bolton & Graham, 2006). Introduction of new jellyfish species is usually characterized by large initial blooms that become less intense; however, the stage is set for subsequent large blooms when fortuitous conditions prevail, and for expansion of the population into new areas (Purcell et al., 2007). The first appearance of *Rhopilema nomadica* was recorded in the Mediterranean in the mid-1970s and now is found along all coastlines of the eastern Mediterranean Sea (Graham and Bayha, 2007). Similarly, the infamous ctenophore, *Mnemiopsis leidyi*, first invaded the Black Sea in early 1980s, then it spread to the Sea of Azov and the Mediterranean and Caspian seas (Purcell et al., 2001c; Graham and Bayha, 2007). Recently, *Mnemiopsis leidyi* appeared in the North Sea and was suspected to be a separate introduction and this introduced species had spread to the Baltic Sea (Faasse and Bayha, 2006; Hansson, 2006). Thus, with introduction of jellyfish species

in different places, blooms may occur in areas where the species did not previously occur (Purcell et al., 2007).

Most of the recorded jellyfish blooms worldwide were attributed to the interaction of multiple contributing factors. For example, blooms in Chinese waters occurred after warming, extensive eutrophication, fishing, construction and invasion (Purcell et al., 2007). Equally, blooms in Mar Menor, Spain were due to the interaction of several factors like eutrophication, construction, modification of bottom habitat from sand to mud, invasion of an algal invader *Caulerpa prolifera* followed by sea grasses replacement by the algal species, introduction of oysters which provides additionl substrate to the jellyfish polyps, hypoxic bottom waters and vigorous fisheries decline (Pagés, 2001). The success of the introduced ctenophore, *Mnemiopsis leidyi*, in the Black Sea was perhaps due to previous ecosystem damage (Oguz, 2005a, b), climate variations (Oguz, 2005a), overfishing (Shiganova, 1998; Daskalov, 2002) and the initial absence of a controlling predator (Purcell et al., 2001c). Table 1 shows the summary of the interacting multiple factors which had contributed to the major jellyfish blooms worldwide.

Table 1. Summary of possible contributing factors to major jellyfish blooms around the world.

Bloom location	Main species	Possible factors contributing to bloom						Source
		Climate	Eutrophication	Fishing	Aquaculture	Construction	Invasion	
Tokyo Bay, Seto Sea	*Aurelia aurita* (s.l)	+	+	+	+	+	+	Ishii (2004), Uye & Ueta (2004)
East Asian marginal seas	*Nemopilema namorai*	+	+	+	?	+	-	Uye (in press)
Yangtze River estuary	*Aequorea sp.,*	+	+	+	?	+	+	Ding and Cheng (2005), Cheng et al. (2005), Xian et al. (2005)
	Cyanea sp.,							
	Sanderia malayensis							
Black Sea	*Mnemiopsis leidyi**	+	+	+	-	+	+	Orguz (2005a,b)
Mar Menor	*Cotylorhiza,*	?	+	+	+	+	+	Pages (2001)
	Rhizostoma							
Mediterranean and	*Pelagia noctiluca**	+	-	+	-	-	-	Goy et al. (1989),
Adriatic seas								Purcell et al. (1999b)
Benguela Current	*Chrysoara hysoscella,*	?	-	+	-	-	-	Lynam et al. (2006)
	Aequorea forskalea							
Chesapeake Bay	*Chrysaora quinquecirrha,*	+	+	+	-	-	-	Purcell and Decker (2005)
	*Mnemiopsis leidyi**							
Bering Sea	*Chrysoara melanaster*	+	-	+	-	-	-	Brodeur et al. (in press)
Lurefjorden	*Periphylla periphylla**	?	-	?	-	-	-	Eaine et al. (1999)

Species indicated with asterisk (*) are holoplanktonic; all others have a benthic stage. + = probable, ? = unknown or not examined, – = unlikely. *Source: adopted from Purcell et al. (2007).*

2.5. Consequences of jellyfish bloom

When jellyfish bloom, they can cause several problems greatly impacting human activities. Lucas et al. (2014) stressed that when jellyfish are abundant, they largely affect the provisioning and cultural ecosystem services. Generally, socio-economic effects like impacts on fisheries and aquaculture (Richardson et al., 2009; Dong et al., 2010; Gibbons and Richardson, 2015; Purcell et al., 2007; Doyle et al., 2008; Nagata et al., 2009;), power and desalination operations (Daryababard and Dawson, 2008; Galil, 2007; Gibbons and Richardson, 2015), tourism industries (Ozturk and İşinibilir, 2010; Cinar et al., 2011; Lucas 2001; Purcell et al., 2007) were reported. Additionally, impacts on human enterprise and health and on the ecosystem, were also documented although rigorous analysis is hampered by a lack of quantitative evidences (Lucas et al., 2014; CIESM, 2001).

2.5.1. Net-based fisheries

The most frequently media or literature-reported problem occurring as a result of increased jellyfish abundance is the intervention with fishing operations (Purcell et al., 2007) and this direct physical interference on net-based fisheries is non-debatable and financially demonstrable (Lucas et al., 2014). Jellyfish blooms result in serious annoyance by (a) clogging and bursting fishing nets, (b) reducing fish harvest, (c) killing and spoiling fish, (d) stinging fishermen as they try to remove jellyfish, (e) increasing the time and labor effort during the removal of medusae from the nets in some instances, (f) causing fishing boats to capsize (Kawahara et al., 2006; Purcell et al., 2007; Uye, 2008; Dong et al., 2010; Quinoñes et al., 2012). The above-mentioned problems are common for Japanese and Korean fisheries located in the Sea of Japan, Yellow Sea and East China Sea, where most set-net fisheries have been negatively affected by blooms of the giant jellyfish *Nemopilema nomurai* and the ubiquitous moon jellyfish, *Aurelia aurita* (Uye, 2008). Following the 2005 bloom, Aomori Prefecture in Honshū, Japan lost two billion JPY (US$ 25 million) while the nationwide loss was estimated to be 30 billion JPY (US$ 380 million) (Uye, 2008).

Blooms of *Lychnorhiza lucerna* also resulted in fishing problems in northern Argentina through reduction of fish captures and catch quality, damaging nets and preventing fishing operations (Schiariti et al., 2008). The Peruvian anchovy fishery (largest single-species fisheries in the world) was seasonally affected by the semaeostome *Chrysaora plocamia*. Blooms of this species in 2008-2009 led to an economic loss of US$ 200,000 in just over one month (Quinoñes et al., 2012). Shrimps fisheries around the globe also suffer from jellyfish blooms. The shrimp fishery in the Gulf of Mexico had suffered from US$ 10 million revenue lost as a consequence of the *Phyllorhiza punctata* (an invasive rhizostome) bloom (Graham et al., 2003). Shrimp fishery in southeastern Brazil also suffered from the year-round bloom of the rhizostome *Lychnorhiza lucerna* through displacement of hauls, as well as clogging of nets (Nagata et al., 2009).

2.5.2. Aquaculture

The impact of jellyfish and ctenophore blooms in the aquaculture industry is evident but not well known (Båmstedt et al., 1998). The blooms of the holoplanktonic *Pelagia noctiluca* and the massive transport of the developed jellyfish into coastal waters and their aggregation around fish farm cages by tidal currents may damage cultured fish (Doyle et al., 2008). Indirect damage to fish cultures can be through hypoxia and following suffocation when there is inadequate water exchange between the cage and surrounding water column. Direct damage can be through stinging of the fish skin and gills as bloomed jellyfish pass through the mesh of the cages, either intact or becoming broken up into smaller pieces (Baxter et al., 2011a; Mitchell et al., 2012).

Lucas et al. (2014) listed some economic losses associated to jellyfish blooms. The costs according to Lucas et al. (2004) are attributed to: (a) direct losses caused by fish mortalities and disposals; (b) reduced growth during or after exposure to harmful agents such as jellyfish, harmful algae, parasites and bacteria; (c) increased operational costs; (d) production losses during emergency slaughtering and the resulting reduced prices and (e) increased insurance premiums. Fish kills in aquaculture pens associated with jellyfish blooms were common problems in Japan

and Scotland (Purcell et al., 2007). In 2007, a major Irish Sea salmon fish kill at Glenarm, Northern Ireland caused a loss of ~ US$ 1.2 million. It was suggested that aquaculture platforms and cages benefited certain jellyfish species such as *Aurelia aurita* and some hydrozoans (Guenther et al., 2009, 2010) by providing a suitable substrate for the settlement and subsequent growth and development of the biofouling polyp phase of the jellyfish life cycle (Lo et al., 2008; Duarte et al., 2013). This aggravated the detrimental effects of hydroids (Guenther et al., 2009, 2010) and jellyfish blooms on aquaculture operations. Reports on jellyfish bloom affecting decapod cultures in India and the USA were also studied (Purcell et al., 2007).

2.5.3. Tourism and human health

The most well-known example of the impact of jellyfish bloom in the society is its damaging effect on coastal tourism. Coastal tourism is one of the world's largest economies and in tropical and subtropical regions, coastal tourism has huge economic importance. Countries like Spain, Portugal, Italy and Greece have economies that depend heavily on tourism, with 130 million visitors, mainly coming from Germany and the UK. Few of the most popular beach destinations in the world include the northern rim of the Mediterranean, the north and north-east coasts of Australia, the Indo-Pacific and the southern United States, (particularly Florida). Several of these regions were seriously and adversely affected by the presence of jellyfish which gave nasty or even fatal stings (*Carukia barnesi, Cyanea lamarckii, Physalia physalis*, *Rhopilema nomadica, Chironex fleckeri*) (Purcell et al., 2007 as cited by Lucas et al., 2014). Commonly, jellyfish may be present in the shallow waters where people swim and snorkel or they may get washed up onto the beaches following strong onshore winds (Lucas et al., 2014).

Serious implications of jellyfish abundance on tourism were reported from Thailand, the Philippines, the northern coast of Australia and other Pacific nations where cubozoan jellyfish were found (Fenner and Williamson, 1996; Fenner et al., 2010). Approximately 10,000 jellyfish sting each summer were caused by *Physalia physalis* on the east coast of Australia. Stings caused by *Catostylus sp*. and *Cyanea sp*.

were also reported (Fenner and Williamson, 1996). Dong et al. (2010), listed the most common causes of jellyfish stings in Chinese coastal waters. The list included *Physalia physalis, Aurelia aurita*, *Nemopilema nomurai*, *Rhopilema esculentum, Cyanea nozakii* and *Pelagia noctiluca*. There were at least 13 known fatalities and several thousand hospitalizations between 1983 and 2007 due to these species.

Gershwin et al. (2010) presented the severe health effects from the sting of the box jellyfish *Chironex fleckeri* and other jellyfish species. Serious effects of envenomation include cardiac and respiratory arrest which may prove to be fatal in only 2–3 min. Recently, Irukandji Syndrome was attributed to *Carukia barnesi* and several other unnamed carybdeids. Signs of the syndrome includes abdominal cramps, nausea, vomiting, intense lower back and chest pain, difficulty in breathing, headache, anxiety and severe hypertension that may last for 1–2 days (Gershwin et al., 2010). Fenner and Hadock, 2002 reported that the first death case from Irukandji Syndrome in Australia occurred in 2002 while Fenner and Harrison (2000) stated that *Chironex* had caused 67 deaths in Australia between 1884 and 1996. In Malaysia and the Philippines, jellyfish related fatalities are far more common, where between 20 and 50 people die each year due to jellyfish stings (Fenner et al., 2010).

2.6. Environmental DNA (eDNA): a new research tool

The first critical phase of biodiversity studies is the assessment of species distribution which is needed for fields like biogeography, conservation biology and ecology (Margurran, 2004). However, species detection which is part of assessing species distribution sometimes becomes difficult, potentially creating biased study results (Gotelli and Colwell, 2001; MacKenzie et al., 2006). For marine species detection for example, conventional sampling tools like nets, grabs, trawls, dredges and corers are requiring a lot of efforts and equipment to use (Sohier, 2013). Some techniques become ineffective when sampling species of low densities. Other limitations of traditional detection and monitoring methods are related with non-standardized sampling procedures, taxonomic identification and the invasive and sometimes destructive nature of some survey techniques. Because of these limitations and difficulties, a new method of species detection using environmental DNA (eDNA) has

recently been developed and seen as an alternative approach for monitoring aquatic ecosystems (Ficetola et al., 2008). This newly immerging detection technique offers a vastly more sensitive, high-throughput, potentially cheaper, less time consuming and less invasive approach to investigate biodiversity as compared with traditional detection techniques (Port et al., 2016).

Environmental DNA refers to small DNA fragments derived from cellular DNA shed by various organisms through their mucus, feces, urine and skin and left behind by the organisms in their environments (Foote et al., 2012; Taberlet et al., 2012a). Sources of eDNA can be microbes and macroorganisms in the environment (Taberlet et al., 2012). Finding eDNA from collected water samples rather than looking for the target species in aquatic environments is considered a relatively fresh and immerging research method (Ficetola et al., 2008, Rees et al., 2014, Thomsen and Willerslev, 2015). The collected eDNA from water samples can provide a snapshot and a record of the species present in the studied environment over the period that the DNA persists in that environment (Hofreiter et al., 2003; Dejean et al., 2011). Procedurally, eDNA technique requires getting sample from the appropriate environment (water or sediment sample for example) followed by the development of genetic markers specific to the desired species. Having developed the genetic markers for the target species, target eDNA fragments can be detected using a variety of molecular methods including traditional or End-Point PCR, and visualization of the PCR product through gel electrophoresis, quantitative or real time PCR (qPCR or rtPCR), Sanger sequencing or the latest Next-generation DNA sequencing (NGS) (Taberlet, et al., 2012b; Yoccoz, 2012). Originally, eDNA approach has been used to study microbial abundance and diversity (Venter et al., 2004; Rusch et al., 2007). Only recently, this technique has been applied to investigate macro and higher eukaryotes like invertebrates (Goldberg et al., 2013; Deiner and Altermatt, 2014; Machler et al., 2014), amphibians (Ficetola et al., 2008; Pilliod et al., 2014), fishes (Thomsen et al., 2012a, b; Jerde et al., 2013) and mammals (Andersen et al., 2012; Foote et al., 2012). Moreover, reports show that eDNA has the power to monitor and quantify rare and endangered freshwater crustaceans, amphibians, insects, fish and mammals. Therefore, the approach has the capacity to

account diversity of the whole lake fauna (Thomsen et al., 2011). Previous macroorganism detection using eDNA has been species specific, however, multi-species PCR in combination with high-throughput sequencing (i.e. metabarcoding) can reveal whole-community eDNA (Port et al., 2016). Despite the increasing number of studies published in the literature employing eDNA technique in surveying and assessing aquatic ecosystems, none has been conducted on the detection of *C. hysoscella* in the North Sea using eDNA. A pioneering study is therefore required to examine the possibility of using this new monitoring approach in studying the abundance and distribution of gelatinous organisms in the North Sea.

Diaz-Ferguson and Moyer (2014) summarized the potential applications of eDNA approach to the fields of marine and terrestrial ecology and conservation biology. Such applications may involve detection of aquatic invasive species (AIS), biodiversity and community assessment, population dynamics and ecosystem health.

Environmental DNA approach can contribute to the growing studies on using molecular methods used for AIS detection. Molecular based AIS detection has become easier through the development of molecular markers specific for the target species. These markers are useful tools for conservation managers that aim to monitor AIS. With eDNA technique, the chance of confirming AIS detection in hours or days instead of weeks or months is possible. This allows managers to respond quickly to limit dispersal and settlement of the invader (Darling and Mahon, 2011). Moreover, AIS detection by eDNA offers clues to determine origin of the introduction and potential routes of invasion (Diaz-Ferguson and Moyer, 2014).

DNA metabarcoding and eDNA approach can be used together to assess biodiversity and community structure of the target environment. According to Taberlet et al. (2012b), DNA metabarcoding is used to perform identification of various species using eDNA. This method depends on NGS, which allows the sequencing of billions of 100 base pair reads, and the creation of taxonomic reference archives which contains sequences for various species (Barcode of Life as an example). In an eDNA metabarcoding approach, it is likely to identify the eDNA of any species or taxon (rather than just identifying a single taxon) collected from a single water sample given that the DNA sequences of these species are already stored in the library. Thus, metabarcoding is a more advantageous tool for

biodiversity assessment as it provides estimates that are rather taxonomically comprehensive, quicker to produce, and less reliant on taxonomic expertise (Ji et al., 2013).

It is also possible to use the detected and quantified eDNA fragments from a sampled environment as baseline or indirect measurement of population characteristics like distribution, biomass and abundance. In fact, it was established that eDNA concentrations are correlated with organism distribution and biomass in freshwater environments (Takahara et al., 2012, 2013). Several authors used eDNA concentration as representation of population distribution of various faunas like amphibians (Ficetola et al., 2008; Goldberg et al., 2011), fishes (Mahon et al., 2012; Minamoto et al., 2012) and reptiles (Piaggio et al., 2013). Not many studies on the correlation of marine eDNA concentration with the species distribution, abundance and biomass have been published in the literature (Diaz-Ferguson and Moyer, 2014). However, temporal and spatial fluctuations of bacterial and phytoplankton communities had been associated with DNA concentration in coastal waters during blooming events (Bailiff and Karl, 1991).

Ecosystem health can be affected by of the presence of AIS and introduced pathogens such as viruses or fungi as these can have serious demographic and genetic impacts to existing native populations (Blanc, 2001). Applications of eDNA approach to monitor virus concentration (Minamoto et al., 2009) or invasive species (Forsstrom and Vasemagi, 2016) help managers to indirectly screen and assess ecosystem health. Additionally, eDNA is a promising tool to investigate changes in community composition and reductions in species diversity, both of which determine ecosystem health (Diaz-Ferguson and Moyer, 2014). It was reported by Strayer (2010) that changes and reduction of species diversity have direct and indirect impacts on the ecosystem as it can reduce water quality, changes nutrient dynamics (Didham et al., 2005) or affect submerged macrophytes distribution (Strayer, 2010). Because of these eDNA potentials, recent claims on the usefulness of eDNA for environmental impact assessments (Veldhoen et al., 2012) and for future risk-based decision making of natural resources (Wilson and Wright, 2013) were published.

Chapter 3: Methodology

Water samples from the study locations established in the BPNS were collected on field from 2014 to 2016. These water samples were immediately filtered onboard RV Simon Stevin to collect the eDNA from the established study locations. The filters were then stored immediately in a freezer to prevent eDNA degradation. From these filters, a selection was made for detection and analysis of the target eDNA. The eDNA was first extracted from the selected filters using CTAB. The quality and quantity of the extracted eDNA was then analyzed using a NanoDrop. The specificity of the designed primer in the study was tested in a PCR reaction and products were evaluated in a gel electrophoresis. With the working primers, the extracted eDNA samples were then subjected to qPCR analysis for estimating concentration of the target eDNA as well as its distribution in the study locations.

3.1. Decontamination of the materials

Following and observing clean laboratory practices in all stages of the eDNA research is important to produce accurate and reproducible results and to avoid contamination (Taberlet et al., 1999). Decontamination was done to remove any DNA present in the work bench and in all the materials used in the experiment. This would also prevent cross contamination in the eDNA experiment. The materials or supplies used in the experiment – from water sampling, eDNA extraction to eDNA detection and amplification were decontaminated prior and after each use. A 10% laboratory-prepared bleach solution was used for the decontamination of all materials as well as the laboratory workplace. Materials were exposed to the bleach solution for 20 minutes and were rinsed completely with deionized water.

3.2. Water sampling for Environmental DNA (eDNA)

Water samples were taken from the nine established study stations in the Belgian part of the North Sea onboard the RV Simon Stevin. Sampling was done in 2014, 2015 and 2016. For this study, selected sampling months for each year were chosen for eDNA extraction: October for 2014; March, May, August and October for 2015 and January, March, May and

August for 2016. Water sampling was done using a carrousel with six Niskin Bottles with CTD (to record conductivity, temperature and depth). The map of the sampling stations is shown in Figure 4. The station's corresponding coordinates and depth are shown in Appendix 1. For surface water samples, the carrousel was lowered to a depth of 3 meters from the surface in every station from which the water samples were collected. After collecting the water samples from the desired depth, the carrousel was pulled up to the surface. Then, two pre-sterilized 1 L bottles (washed with 10% bleach solution followed by cleansing with deionized water) were used to collect the sampled water from the Niskin bottles. Prior to this, the clean bottles were rinsed with a small portion of seawater from the Niskin bottles. After rinsing, the bottles were filled with the collected water samples. For each station and period of sampling, two 1 L bottles were filled with the collected water samples. Each bottle corresponded to the replicate of the water sample per station.

The collected water samples in the 1 L bottles of each station were immediately filtered on board using a magnetic filter funnel (Pall). The membrane filters were made from polyethersulfone (47 mm diameter, Pall) with 0.45 µm pore size. After filtration, the filter was placed in a 2 mL Eppendorf tube. The Eppendorf tubes were labeled with the name of the sampling station, sampling depth and date of sampling. The filtration procedures were repeated for the other replicates of the collected water samples. The filters were then stored at -20°C to prevent DNA degradation. They were then transported to the laboratory using a coolbox

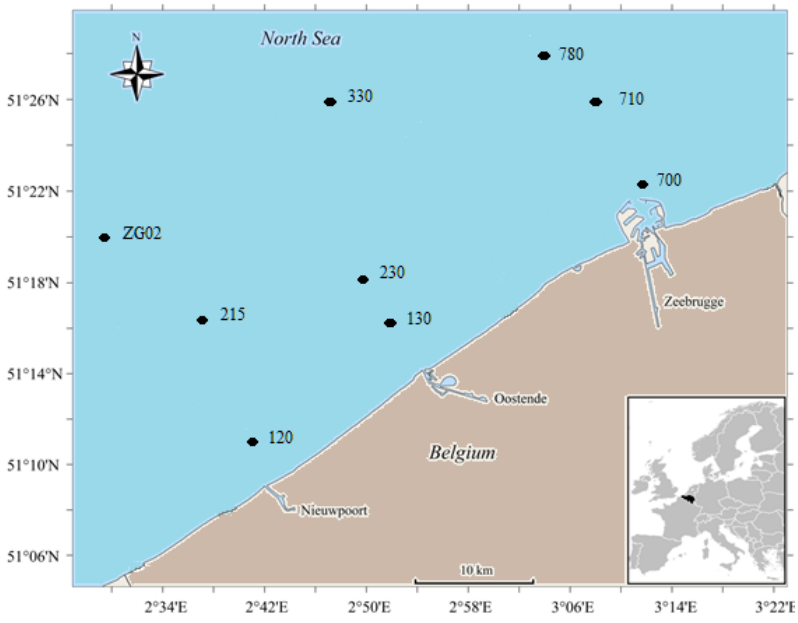


Figure 4. The map showing the locations of the nine study stations of the present study in the Belgian part of the North Sea.

with freezer blocks or ice and stored in the laboratory freezer (-20^{o}C) until eDNA extraction started.

3.3. eDNA extraction

The previously stored filtered samples (the filters) were removed from the freezer (-20^{o}C) and brought to the workbench. Using dissecting knife and forceps, the filter paper for each sampling station was equally divided into six sections. Each section was carefully and separately placed into an autoclaved and pre-labelled 2 mL Eppendorf tubes. For each round of eDNA extraction, six samples were worked on. It was made sure that all the residues from the filter paper were placed into the tubes to maximize the amount of eDNA collected in the tubes. The dissecting materials were washed with 10% bleach solution and then subsequently with deionized water before using it for the next sample to avoid contamination. Then, clean glass beads were placed into each Eppendorf tube containing the cut filter paper. After this, 300 µL of freshly prepared and warm CTAB extraction buffer (65^{o}C) was introduced into each Eppendorf tube using an appropriate pipette (see eDNA extraction protocol for CTAB preparation in Appendix 2). The tubes were then locked properly and then the content was mixed using the MS1 Minishaker for about one minute. During the mixing period, the cut filter paper was made sure to be completely immersed into the extraction buffer. After vortexing, the tubes were warmed by placing them into the heat block (65^{o}C) for one hour and were quickly vortexed every 20 minutes. After heating, the tubes containing the filter material were placed into an Eppendorf tube rack and were placed under a fume hood. Then, 300 µL of phenol:chloroform:Isoamylalcohol (P:C:I) was pipetted in each tube. When pipetting the P:C:I, the top aqueous layer was avoided and only the bottom layer was pipetted. The tubes containing the P:C:I were then vigorously vortexed to mix until the filter paper inside the tubes was dissolved in the solution.

Next, the tubes were then spun at 15 000 rcf for 20 minutes in a cooled (4^{o}C) microfuge (Mikro 200R Hettich Zentrifugen). After centrifuging, two layers of liquid became visible inside the tubes. The supernatant (the clearer upper layer) was carefully pipped out from the 2 mL tube using the appropriate pipette and transferred into an autoclaved and pre-labeled 1.5 mL Eppendorf tube. During pipetting, all the supernatant was removed and the

debris (the dirt below the supernatant) was left behind and not drawn together with the clear liquid. Then, each 1.5 mL Eppendorf tube containing the supernatant was pipetted with 5 µL RNase (Qiagen, 19101). The tubes were incubated on the bench top for 20 minutes. After incubation, the tubes were again brought under the hood and each was pipetted with 500 µL chloroform:isoamylalcohol and were subsequently centrifuged at 15 000 rcf for 15 minutes at room temperature (22^{o}C). After centrifuging the tubes, the supernatant (the clear top layer of solution) was again transferred into autoclaved and pre-labeled 1.5 Eppendorf tubes. Again, the scum or milky dirt layer below the supernatant was avoided and was not pipetted. Each of the tube containing the supernatant was then pipetted first with 27 µL of 3 molar sodium acetate and then with 500 µL of 2-propanol. Each tube was hand-inverted three times to mix and was incubated on the bench top for 10 minutes.

After this, the samples were spun at 10 000 rcf, 4^{o}C for 15 minutes (or more) until pellets were observed at the bottom of the tubes. After pellet formation, the 2-propanol was then slowly pipetted out the tubes and discarded. Extra careful was observed to not include the pellet during pipetting. After removing the 2-propanol from all the tubes, 500 µL of 70% ethanol was then pipetted into each tube to wash the pellet. Then, the tubes were centrifuged at 10 000 rcf, 4^{o}C for 15 minutes. Then, the ethanol was completely removed in a similar manner the propanol was previously removed. Then, for the second time, 500 µL of 70% ethanol was added to each tube then all the tubes were centrifuged again in 1000 rcf, 4^{o}C for 15 minutes. Finally, the ethanol was removed after centrifugation. The pellets were then air-dried until the methanol was completely evaporated from the tubes (maximum 1 hour of air drying). After drying, the first tube (of the four tubes) of each sample was re-suspended with 30 µL of 1X TE buffer. The pellet was allowed to dissolve in the buffer solution by slowly pipetting the solution up and down. The TE buffer used in the first tube was used to re-suspend all the filterpieces of the remaining tubes coming from the same sample.

3.4. Measuring the purity of extracted eDNA: NanoDrop

After eDNA extraction, the quality or the purity of the DNA extracts were determined using a NanoDrop. Prior to sample measurement, the blank (1X TE buffer) was measured first. To start blank measurement, 1.2 µL of 1X TE buffer was dropped on the center hole of the Nanodrop's pedestal. After this, the pedestals were cleaned and then 1.2 µL of the DNA extract was pipetted in the pedestal for sample measurement. During pipetting of the samples into the pedestal, it was made sure that bubbling of the pipetted samples was avoided (see NanoDrop manuals online). After eDNA quality determination, completely labeled samples were then stored in the freezer (-20^{o}C) for future use.

3.5. Testing primer specificity: PCR

Prior to qPCR analysis of the *C. hysoscella* eDNA samples, forward and reverse primers for the target DNA were initially designed (see PCR/PCR primer design protocol in Appendix 3). The primers used in the present study were pre-designed by Tara Grosemans and were made available prior to the start of the PCR/qPCR reactions. Details about the designed primers are shown in Table 2. The information of the amplicon product is also shown.

Table 2. Primer information for the used *C. hysoscella* primers used in the study.

Primer	Start	Stop	Length	Tm	GC%
Forward	77	98	21	63	47.6
CCCAGGAATGACAATGGACAA (Sense)					
Reverse	143	171	28	62	35.7
CCCTGCTAATACAGGTAAAGATAATAGT (AntiSense)					

Amplicon *C. hysoscella*: **Amplicon length: 94**

TTAGTTTACATTGTGCGGGTGCCTCCTCCATTATGGGGGCTATTAATTTTATTACTACTATCTTAAAT
ATGAGAGCCCCAGGAATGACAATGGACAAAATCCCTCTGTTTGTTTGGTCCGTTTTAATTACAGCT
ATCTTATTACTATTATCTTTACCTGTATTAGCAGGGGCGATTACGATGTTATTGACAGACAGAAATT
TTAATACTACATTCTTTGAACCCCCAAGGGGGAG

Figure 5. *C. hysoscella* amplicon product used in the study.

The designed forward and reverse primers for the experiment were received in lyophilized form. Hence, the primers were processed and 100 µL of 10 µM of working primer stock (for forward and reverse primers) were prepared (see PCR protocol in Appendix 4 for details). Before testing the designed primers on the eDNA samples of the present study, its specificity was first tested using eight DNA extracts of various jellyfish species (*Aurelia aurita, Hydrozoa sp., Beroe sp., Cyanea lamarkii, Aurelia vikrina, Mnemiopsis leidyi, Eutonia indicans*) which included a positive control (*C. hysoscella* DNA extract). A negative control which was composed of a solution of PCR reaction reagents (with no DNA extract) was also included. Each PCR reaction tube contained: 2 µL DNA, 1 µL of forward primer (10 µM), 1 µL of reverse primer (10 µM) 6 µL of water and 10 µL of master mix (Roche FastStart Essential Green Master mix 2.0X) and was run on the following conditions: 95^{o}C for 10 minutes; 50 cycles of 95^{o}C for 30 seconds, 50^{o}C for 30 seconds and 72^{o}C for 20 seconds; 95^{o}C for 10 minutes; 65^{o}C for 60 seconds; 97^{o}C for 1 second.

3.6. Verification of primer specificity: gel-electrophoresis

To check if the previously prepared working primer stock solutions were properly prepared and if these primers demonstrate specificity by binding only to the target DNA fragments (which results to its amplification), PCR products were analyzed on a gel electrophoresis. This step visualized the amplified target DNA as DNA bands in the gel. Prior to running the gel electrophoresis, the buffer solution was first prepared. 5X TBE was prepared by weighing 54 g Tris base and 27.5 g boric acid. Both were then transferred in a 1000 mL beaker and then dissolved with 900 mL deionized water. Then, 20 mL of 0.5 M EDTA (pH 8) was added to the solution and the solution was adjusted to a final volume of 1 L. From the 5X TBE, 1X TBE was prepared and used as the buffer solution for the gel electrophoresis.

Before running a gel electrophoresis, the gel tray, comb and buffer tank were rinsed with ethanol followed by deionized water. These were again rinsed with ethanol for final cleaning and then air dried to remove the ethanol. The gel tray was then clamped into its holder and the comb was inserted into the gel tray. After this, 0.9 g of agarose was weighed in a 200 mL beaker. The beaker with the agarose was then added with 60 mL of 1X TBE. The

mixture was then melted in the microwave for one minute in a power stand of seven. After melting, the beaker was carried under the hood to cool down to 60^{o}C for 5 minutes. After cooling, 6 µL of GelRed was carefully added to the melted agar wearing purple nitrile gloves and using the dedicated pipette and pipette tips for this purpose. The beaker was then gently swirled to mix the GelRed into the gel solution. The gel was then gently poured into the gel tray and let to solidify for about 15-20 minutes.

While waiting for the gel to solidify, the PCR products were prepared in 0.2 mL Eppendorf PCR tubes. In each tube 3 µL of the PCR product (DNA extract), 2 µL of molecular grade water and 1 µL loading dye was pipetted. The content of the tube was mixed by slowly pipetting it up and down. The tubes were then spun in a Galaxy Ministar vortex for about 5 seconds to collect all the liquid at the bottom of the tube. After preparing the PCR products, gel tray was placed into the buffer tank with the gel wells facing the positive electrode of the tank. The tank was then filled with 1X TBE buffer until the gel was completely immersed into the buffer solution. It was made sure that the wells were also filled completely with the buffer solution. Loading the gel with the DNA extracts followed. First, 6 µL of the DNA ladder was carefully loaded in the first gel well. Same volume of the PCR products was loaded to the remaining wells. The gel was then run for 10 minutes at 50V then for 60 minutes at 100V. When the bromophenol blue migrated for about 2/3 in the gel towards the negative pole, the power source was turned off to stop the gel electrophoresis.

After running the gel electrophoresis, the gel was scanned using a UV-light source to see the distribution of the DNA bands into the gel. Working primers which amplified the target DNA from the positive control (*C. hysoscella* DNA extract) should show DNA bands on the well where the target DNA extract was injected and bands should appear approximately on the 90-100 base pair DNA ladder fragments (see gel electrophoresis protocol in Appendix 5). After scanning the gel for amplified products, the gel tray, comb and buffer tank were cleaned using soap in a plastic bucket used for this purpose. Then, these materials were rinsed with deionized water and then dried using a paper towel. All the washings were disposed to a container which collects all the liquid wastes from the gel electrophoresis procedure.

3.7. Detection and quantification of *C. hysoscella* eDNA from the samples: qPCR

The qPCR analysis of the eDNA samples was done in the Marine Research Station of VLIZ in Oostende. Prior to qPCR analysis, a series of $1/10^{th}$ standard dilutions (STD) of the *C. hysoscella* amplicon (see Figure 6) with an initial concentration of 10 ng/µL were made. Then, the working primer stocks were prepared in the similar manner as how it was done for the PCR reaction. The master mix was then prepared for the qPCR. To prepare the master mix needed for every qPCR cycle using a 96-well plate, exactly 576 µL of molecular grade water was pipetted in the 2 mL Eppendorf tube. This was then pipetted with 96 µL of the reverse working primer and with 96 µL of forward working primer. Finally, 960 µL of DNA master green (2.0 X) was added to the tube. The master mix was then slowly mixed by pipetting it up and down. The master mix tube was then wrapped with aluminum foil to prevent photodegradation of its sensitive components.

After preparing the master mix solution, the 96-well plate for qPCR was made ready in the workbench together with the template (see Figure 6) which shows the position of the STDs, controls and eDNA samples in the plate. Then, 18 µL of the master mix was pipetted into each well of the plate using an Eppendorf Multipipette Stream. These wells were then added either with 2 µL of the appropriate STD (=standard), 2 µL of the sample, 2 µL of DNA green master molecular grade water (NTC= no template control) and 2 µL *C. hysoscella* DNA extract (positive control) giving a total qPCR reaction volume of 20 µL. The positive control was included to check if the working primer solution used in the qPCR contained the designed primer and if the primers worked well. The negative control (NTC) was included to monitor contamination during the qPCR reaction. The STDs, eDNA samples, negative control and the positive control were all replicated three times in the wells of the plate. After loading the wells, the plate was covered with an adhesive film. Care was taken to not touch the part of the film that will cover the plate. The film was held only on its edge to avoid contamination. A plastic spatula was then used to tightly press the film over the plate. The plate was then spun down for few seconds in the PCR plate spinner to spin down the contents of the plates and to eliminate bubbles from the solution inside the plate. The plate

was then loaded in the qPCR machine (LightCycler® 96, Roche) and run for two hours in the same conditions previously mentioned in the PCR reaction.

LightCycler® 96

Experiment: 20170323_C_Hysoscella_Field_14-15.lc96p

Plate Id: <None>

*	1	2	3	4	5	6	7	8	9	10	11	12
A	S std 10 None [1.000E-9]	S std 10 None [1.000E-9]	S std 10 None [1.000E-9]		U 700 OCT 2014 None	U 700 OCT 2014 None	U 700 OCT 2014 None	- NTC None	- NTC None	U 700 MAR 2015 None	U 700 MAR 2015 None	U 700 MAR 2015 None
B	S std 11 None [1.000E-10]	S std 11 None [1.000E-10]	S std 11 None [1.000E-10]		U 710 OCT 2014 None	U 710 OCT 2014 None	U 710 OCT 2014 None	- NTC None	- NTC None	U 710 MAR 2015 None	U 710 MAR 2015 None	U 710 MAR 2015 None
C	S std 12 None [1.000E-11]	S std 12 None [1.000E-11]	S std 12 None [1.000E-11]		U 780 OCT 2014 None	U 780 OCT 2014 None	U 780 OCT 2014 None	- NTC None	- NTC None	U 780 MAR 2015 None	U 780 MAR 2015 None	U 780 MAR 2015 None
D	S std 13 None [1.000E-12]	S std 13 None [1.000E-12]	S std 13 None [1.000E-12]		U 130 OCT 2014 None	U 130 OCT 2014 None	U 130 OCT 2014 None			U 130 MAR 2015 None	U 130 MAR 2015 None	U 130 MAR 2015 None
E	S std 14 None [1.000E-13]	S std 14 None [1.000E-13]	S std 14 None [1.000E-13]		U 230 OCT 2014 None	U 230 OCT 2014 None	U 230 OCT 2014 None			U 230 MAR 2015 None	U 230 MAR 2015 None	U 230 MAR 2015 None
F	S std 15 None [1.000E-14]	S std 15 None [1.000E-14]	S std 15 None [1.000E-14]		U 330 OCT 2014 None	U 330 OCT 2014 None	U 330 OCT 2014 None			U 330 MAR 2015 None	U 330 MAR 2015 None	U 330 MAR 2015 None
G	+ C hysoscella None	+ C hysoscella None	+ C hysoscella None		U 215 OCT 2014 None	U 215 OCT 2014 None	U 215 OCT 2014 None			U 120 MAR 2015 None	U 120 MAR 2015 None	U 120 MAR 2015 None
H	- NTC None	- NTC None	- NTC None		U ZG02 MAR 2015 None	U ZG02 MAR 2015 None	U ZG02 MAR 2015 None			U 215 MAR 2015 None	U 215 MAR 2015 None	U 215 MAR 2015 None

Figure 6. Plate template or guide generated through LightCycler® 96 Roche. Blue blocks (STDs) represent standard dilutions, pink blocks (NTC) represent negative controls, green blocks (*C. hysoscella*) represent positive controls and grey blocks represent eDNA samples.

Chapter 4: Results

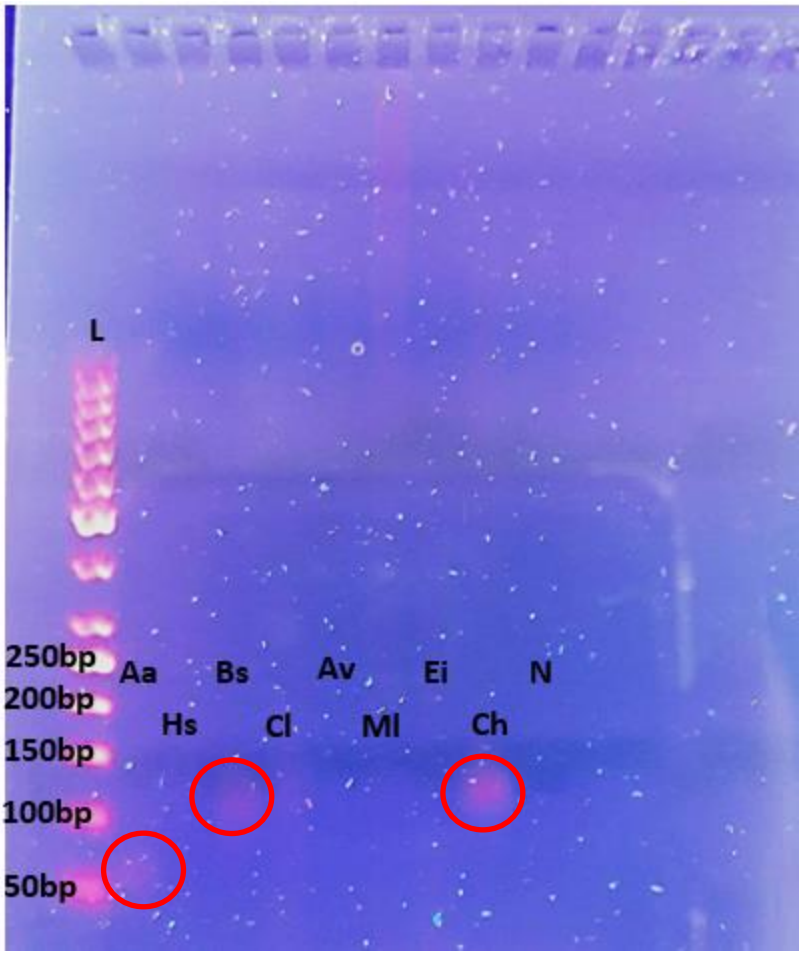
4.1. eDNA extraction

The eDNA samples collected from the different sampling stations of the considered sampling periods were extracted from the filter materials using the CTAB protocol. The extracted DNA was then measured using a NanoDrop. The eDNA concentration (ng/µL), A260/280 and A260/230 ratios were measured. For the extracted eDNA samples to be considered in good quality and purity their A260/280 ratio should be between 1.7 to 2.0 and the A260/230 ratio should be 2.0 or greater. The A260/280 ratio is generally used to determine protein contamination in the nucleic acid sample. A lower ratio indicates the sample is protein contaminated. The presence of protein contamination may have an effect on downstream applications that use the nucleic acid samples such as PCR and qPCR. The A260/230 ratio indicates the presence of organic contaminants. Samples with 260/230 ratios below 1.8 are considered to have a significant amount of these contaminants that will interfere with downstream applications. A total of 76 eDNA samples were extracted and analyzed in the study. Form these extracts, 30 met the standards for 260/280 and 260/230 ratios, 25 met the standards for 260/280 but has 260/230 ratios below the standards, 2 met the standards for 260/230 but has 260/280 ratios below the standards extracts and 19 extracts have both ratios below the ideal values. All the extracted samples were analyzed in the qPCR. The quality of the eDNA extract per station is shown in Table 5. The details of the NanoDrop measurements of the extracted eDNA samples are presented in Appendix 6.

4.2. *C. hysoscella* primer specificity

Before the qPCR analysis of the extracted eDNA samples using the designed *C. hysoscella* primer in this study, the primers' specificity to the target DNA was first verified using tissue DNA extracts of different jellyfish species namely: *Aurelia aurita, Hydrozoa sp., Beroe sp., Cyanea lamarkii, Aurelia vikrina, Mnemiopsis leidyi* and *Eutonia indicans* through PCR reaction. Tissue DNA extracts of *C. hysoscella* was used as the positive control and a solution of PCR reaction reagents (with no DNA extract) was included as the negative

control. The PCR products were run in a gel electrophoresis to verify specific amplification of the target DNA. Figure 7 below shows the result of the gel electrophoresis. The result of the gel electrophoresis showed specific amplification of the *C. hysoscella* target DNA using the designed primers resulting to an amplicon product of approximately 100bp. However, as seen on the gel electrophoresis, slight/weak DNA bands were observed for *A. aurita* (approximately 60bp) and *Beroe sp*. (approximately 100bp) tissue extracts. These very small amounts of amplified products were suspected to be primer dimers formed during the PCR reaction in case of *A. aurita*. In the case of *Beroe sp*., it could be that the primer binds to a similar but not identical part in the genome of *Beroe sp*. But no molecular or genomic information on this species is available, so it is not possible to confirm this claim.



Legend: L = 50bp DNA ladder; Aa = *A. aurita*; Hs = *Hydrozoa sp*.; Bs = *Beroe sp*.; Cl = *C. lamarkii*; Av = *A. vikrina*; Ml = *M. leidyi*; Ei = *E. indicans*; Ch = *C. hysoscella* DNA extracts; N = negative control

Figure 7. Gel electrophoresis of the PCR products from tissue DNA extracts of various jellyfish species using the designed *C. hysoscella* primers used in the study.

4.3. qPCR analysis of eDNA samples

qPCR of the eDNA extracts was made to determine the presence or the absence of the *C. hysoscella* eDNA from the water samples collected from the different study locations during the sampling periods considered. If present, qPCR enables the estimation of the amount of the target eDNA originally present in the analyzed samples.

4.3.1. Presence or absence of *C. hysoscella* eDNA

The presence or absence of the *C. hysoscella* eDNA from the nine studied stations in the BPNS was evaluated through the qPCR of the extracted samples. The results of the qPCR analysis for detection and non-detection of the target eDNA is presented in Table 3. As reflected on the table, *C. hysoscella* eDNA is commonly present in the studied stations during October 2014 (6 out of 7 sampled stations), March 2015 (present in all stations), May 2015 (6 out of 8 sampled stations) and August 2015 (6 out of 8 sampled stations).

Table 3. Detection and non-detection of *C. hysoscella* eDNA in the established study locations in the BPNS.

Sampling time	Stations								
	120 (S)	**130 (S)**	**700 (S)**	**215 (M)**	**230 (M)**	**710 (M)**	**ZG02 (OS)**	**330 (OS)**	**780 (OS)**
Oct-14	NS	1	1	1	1	0	NS	1	1
Mar-15	1	1	1	1	1	1	1	1	1
May-15	1	0	0	NS	1	1	1	1	1
Aug-15	0	1	1	0	1	0	1	1	1
Oct-15	0	NS	0	1	0	0	1	1	0
Jan-16	0	0	0	NS	1	0	1	1	0
Mar-16	0	1	0	1	1	0	1	1	0
May-16	0	0	0	1	0	0	1	0	0
Aug-16	1	0	0	0	1	1	1	0	0

Note: NS – no sample analyzed; 1 – detected; 0 – not detected; S – shoreline stations; M – middle stations; OS – offshore stations; 120, 215 and ZG02 are Nieuwpoort stations; 130, 230 and 330 are Oostende stations; 700, 710 and 780 are Zeebrugge stations.

The occurrence of the target eDNA for the selected months in 2016 decreased. For 2016, the target eDNA is frequently present during March (5 out of 9) and August (4 out of 9). Table 3 also shows that *C. hysoscella* eDNA is commonly detected in Oostende stations then in the Niewpoort stations and least detected in Zeebrugge stations. Additionally, the target eDNAs are commonly present in stations farther from the shoreline (see Figure 4).

4.3.2. Tracing the possible cause of false positive results

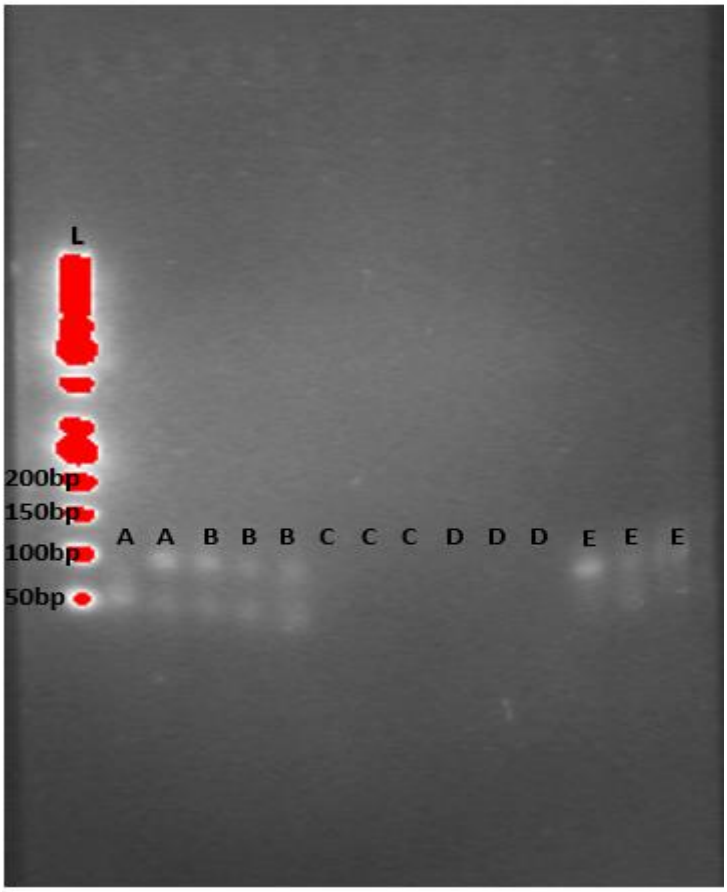
Some of the NTCs did result in a positive amplification during the qPCR analyses done in the study. To determine whether this was a false positive, potential primer dimerization or contamination, a verification test was done. The same master mix used in the qPCR analysis was run in the PCR. This test also run a PCR for the used positive control (*C. hysoscella* tissue sample 249 extract and dilutions of such extract); it additionally included a no primer PCR reaction solution and the same no template control PCR reaction solution. After the PCR, each PCR product was run in the gel electrophoresis in three replicates except for the tissue sample which was tested in two replicates to allow the best use of the available wells in the gel. The components run in the PCR are shown in Table 4. The result of the gel electrophoresis is shown in Figure 8.

Table 4. Solutions used in the qPCR analysis tested for contamination in the gel electrophoresis.

Well	Master mix (uL)	Primer (uL)	DNA (uL)	Water (uL)	Content
1	-	-	-	-	50bp DNA ladder
2	10	2	2	6	*C. hysoscella* tissue sample 249
3	10	2	2	6	
4	10	2	2	6	1:1 *C. hysoscella* tissue sample 249 (1/2 dilution)
5	10	2	2	6	
6	10	2	2	6	
7	10	0	0	10	Master mix
8	10	0	0	10	
9	10	0	0	10	

10	10	0	2	8	No primer
11	10	0	2	8	
12	10	0	2	8	
13	10	2	0	8	No template control (NTC)
14	10	2	0	8	
15	10	2	0	8	

The figure reveals that the master mix used in the qPCR analysis was not contaminated by the target DNA as samples containing only mastermix did not result in amplification (wells C in the figure). Also, based from the gel analysis below, no amplified products were formed for PCR reaction solutions that were not added with *C. hysoscella* primers (wells D in the figure) while amplified products of approximately 100bp were produced in the NTCs.



Legend:
L = 50bp DNA ladder; A = *C. hysoscella* tissue sample 249; B = 1:1 *C. hysoscella* tissue sample 249 (1/2 dilution); C = Master mix; D = No primer; E = No template control (NTC)

Figure 8. Gel electrophoresis of the components tested in Table 4.

4.3.3. Cq means of the eDNA samples

The quantitation cycle (Cq) means as measured through the qPCR of the extracted eDNAs from each sampling station and sampling time are presented in Table 5. Each extracted sample was replicated three times in the qPCR plates. If the target eDNA is present in each replicate, a Cq value for each replicate is given. The mean of these values is taken into account. Cq means are chosen to represent the abundance or concentration of the target eDNA since the estimated concentrations based from the qPCR analysis are very small and the difference in the magnitudes of the estimated values are great (see Appendix 7). Hence, graphical representation of the results would be better using the raw Cq means, as these are values on a log scale, rather than doing a transformation of the data. In qPCR, the cycle in which fluorescence can be detected is represented by Cq. The fluorescence on the other hand gives an idea on the initial amount of the target eDNA in the sample. The higher the initial number of the target eDNA in the sample, the faster the increase in the fluorescence during the qPCR cycles. Therefore, lower Cq values mean higher initial amount of the target eDNA while higher Cq values mean lower initial amount of the target eDNA.

Table 5. Cq means obtained from the qPCR analysis of the extracted eDNA samples.

Sampling time	Station and Cq mean								
	120 (S)	130 (S)	700 (S)	215 (M)	230 (M)	710 (M)	ZG02 (OS)	330 (OS)	780 (OS)
Oct-14	NS	34.13	29.46	29.19	32.46	-	NS	30.25	33.19
Mar-15	32.65	40.34	39.91	31.01	37.63	35.93	31.90	32.12	31.52
May-15	43.38	-	-	NS	40.11	39.88	44.66	41.41	40.23
Aug-15	-	33.78	41.58	-	44.51	-	40.83	30.10	38.86
Oct-15	-	NS	-	41.30	-	-	42.50	42.41	-
Jan-16	-	-	-	NS	40.52	-	44.04	37.33	-
Mar-16	-	41.04	-	40.02	38.86	-	43.40	40.10	-
May-16	-	-	-	41.61	-	-	43.06	-	-
Aug-16	44.98	-	-	-	40.50	2.35	39.77	-	-

Note: NS – no sample analyzed; – means non-detection; S – shoreline stations; M – middle stations; OS – offshore stations; 120, 215 and ZG02 are Nieuwpoort stations; 130, 230 and 330 are Oostende stations; 700, 710 and 780 are Zeebrugge stations; green – 260/280 and 260/230 ratios are ok; yellow – 260/280 ratio is ok but 260/230 not ok; blue – 260/280 ratio not ok but 260/230 ok; red – both 260/280 and 260/230 ratios are not ok.

Based from the Cq means in Table 5, *C. hysoscella* eDNA is usually in greater abundance in the middle stations (215 for October 2014, 215 for March 2015, 710 for May 2015, 215 for October 2015, 230 for March 2016, 215 for May 2016 and 710 for August 2016) and in the offshore stations (330 for August 2015 and January 2016). From the three middle stations, higher amount of the target eDNA is frequently found in the Nieuwpoort station across the sampling months and with an occasional detection of increased amount of eDNA in Oostende station and in Zeebrugge station. For the offshore stations, higher abundance is commonly observed in Oostende station. Very low detection to non-detection of the *C. hysoscella* eDNA is common in the shoreline stations across the sampling months. The abundance of the target eDNA in the shoreline stations has decreased in 2016 as compared to the previous years. Additionally, as seen in Table 5, the target eDNA started to disappear from October 2015 onwards in all Zeebrugge stations except for a sudden re-appearance and increase in abundance in station 710 during August 2016. In the Nieuwpoort stations, *C. hysoscella* eDNA becomes commonly detected when moving away from the shoreline. In the middle and offshore Nieuwpoort station, there is an increase-decrease trend of the target eDNA across the sampling months. However, there is a noticeable increase in the abundance of the eDNA in the offshore station (ZG02) in August 2016 as compared to the previous months (except March 2015). A similar increase-decrease trend is observed in all Oostende stations across the sampling months. However, in August 2016, the target eDNA is detected only in Oostende in the middle station (230) in low amount.

4.3.4. Spatial and temporal variation in the eDNA distribution

The Cq values from the qPCR analyses were used to see if there is a spatial and temporal variation in the distribution of the *C. hysoscella* eDNA. To evaluate a possible temporal variation across stations, the average of the Cq mean per station across the sampling times was considered. For spatial variation within the same sampling period, the average of the Cq mean of all stations per sampling time was considered. The standard deviation of the Cq mean in a station across the sampling times and that of all stations per sampling time was also calculated. These are presented in Table 6.

Table 6. Average of the Cq means and the standard deviation of the Cq means per month and per station.

Sampling time	Average Cq mean per month	Standard deviation of the Cq mean per month	Station	Average Cq mean per station	Standard deviation of the Cq mean per station
Oct-14	31.45	2.09	**120**	40.34	6.70
Mar-15	34.78	3.73	**130**	37.32	3.90
May-15	41.61	1.98	**700**	36.98	6.57
Aug-15	38.28	5.36	**215**	36.63	6.02
Oct-15	42.07	0.67	**230**	39.23	3.66
Jan-16	40.63	3.36	**710**	26.05	20.62
Mar-16	40.68	1.70	**ZG02**	41.27	4.12
May-16	42.34	1.03	**330**	36.25	5.35
Aug-16	31.90	19.83	**780**	35.95	4.24

The data in Table 6 are presented in Figures 9 and 10. The figures show that there are some spatial and temporal variations in the amount of *C. hysoscella* eDNA as predicted in this study. Although the average of the Cq means across all stations per sampling months differ, the values do not follow the exact same pattern as the changes in temperature (see Figure 9). Nevertheless, it is important to mention that Figure 9 reveals that the average Cq mean from all stations for the months of

October 2014, March 2015, August 2015, and August 2016 are lower compared to the other sampling months. This indicates that eDNA abundance are a bit higher in these mentioned sampling months. From these months, lowest Cq mean (highest eDNA abundance) is recorded for August 2016. The average temperature of all sampling stations per sampling month is also plotted in the graph to see if temperature influences the abundance of the target eDNA across the different sampling months (see Appendix 8 for the temperature profile of all the sampling stations per sampling month).

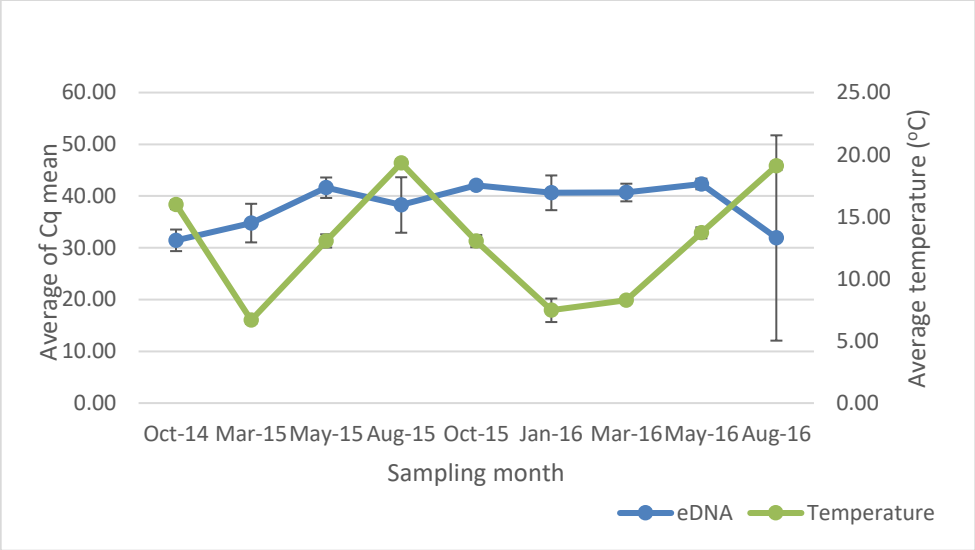


Figure 9. eDNA abundance and temperature across sampling months.

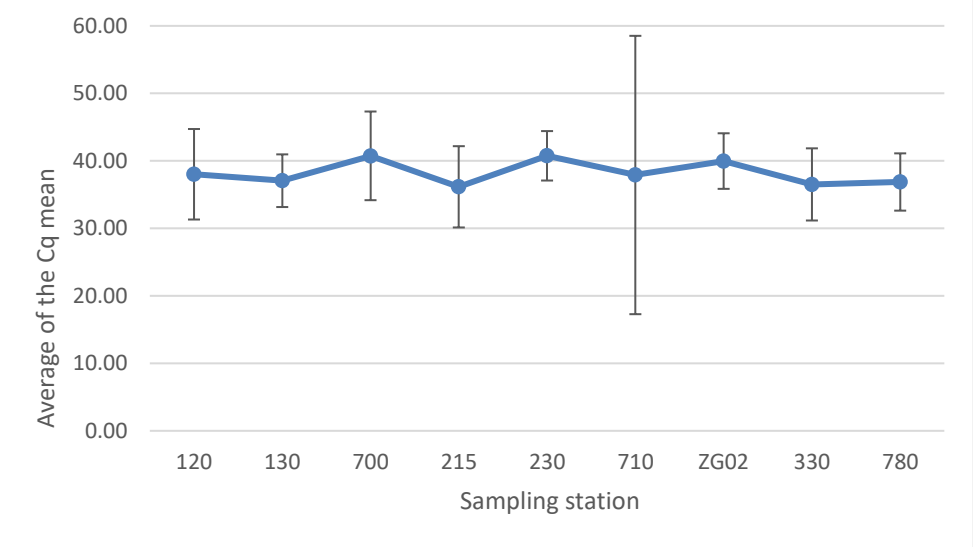
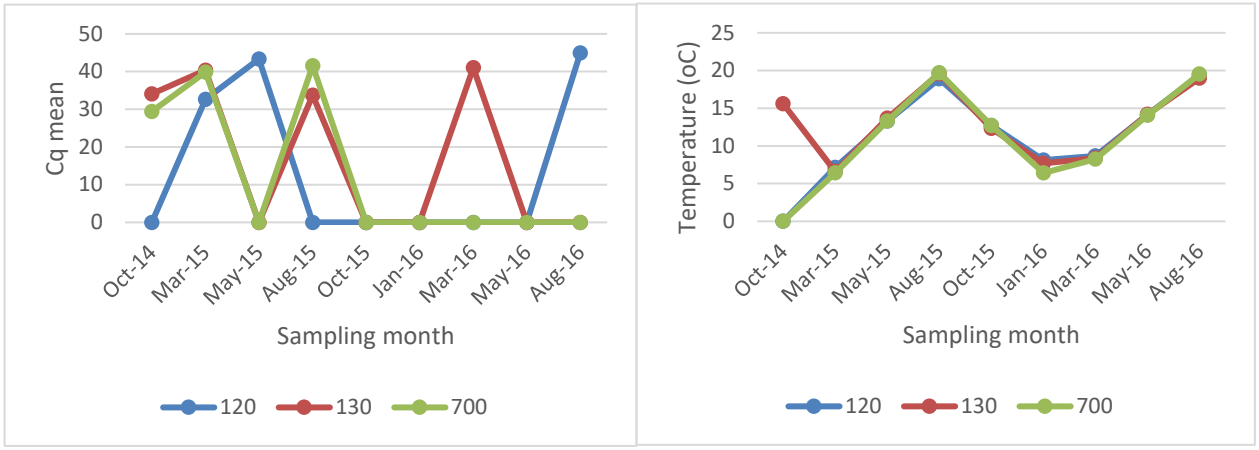


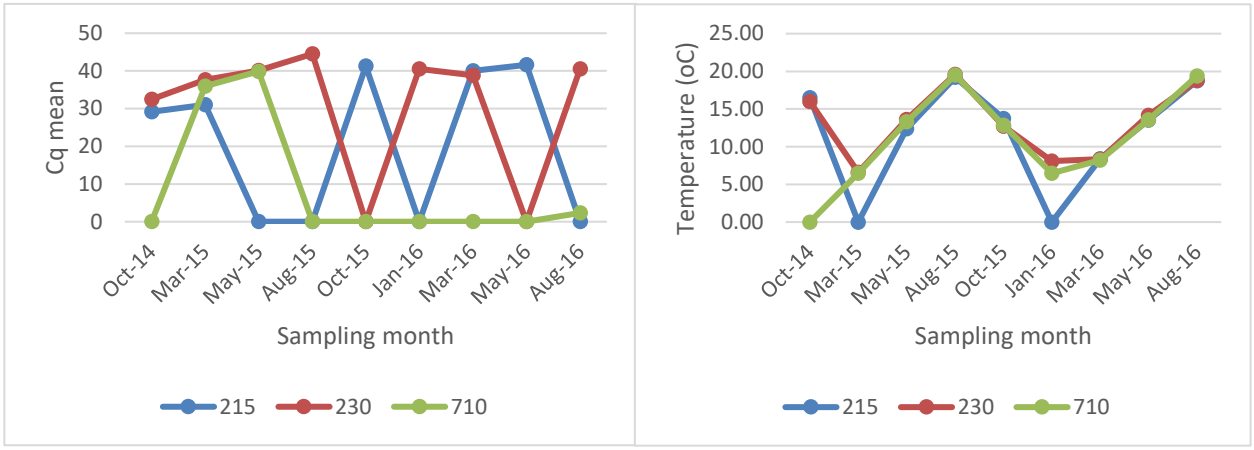
Figure 10. eDNA abundance across sampling stations.

Figure 9 shows that there is no relationship between the average temperature across all stations per sampling month and the abundance of the target eDNA. This is confirmed by a correlation test performed between the Cq means and temperatures from all stations from the different sampling months ($p = 0.4254$). Figure 10 reveals that the amount of *C. hysoscella* eDNA detected per station across the sampling months does not vary very much. However, the Cq mean for station 710 is obviously lower compared to the other stations. This implies that throughout the sampling periods considered, highest *C. hysoscella* eDNA is found in this station.

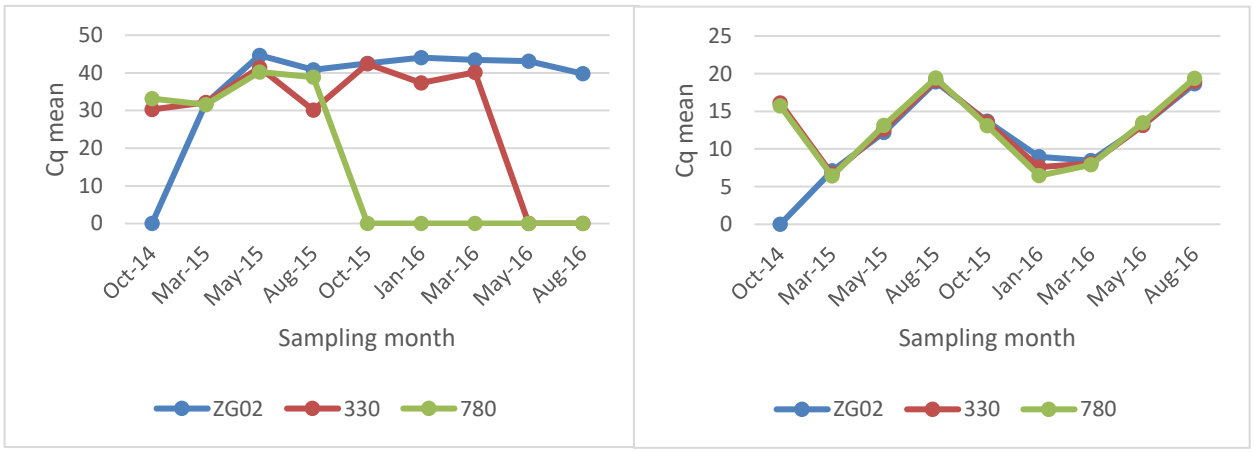
To see the fluctuations or the trends in the change in the abundance of *C. hysoscella* eDNA in stations near the shoreline, in the middle stations and in the offshore stations, Figure 11 is presented. The trend in the temperature per station is also shown. Clearly, the trends in the temperature do not affect the trends in the Cq mean (or *C. hysoscella* eDNA abundance). There is no consistent and clear trend on the changes in the *C. hysoscella* eDNA abundance across the sampling months per station. However, based from the figure, it is clear that from start of the sampling time (October 2014), the target eDNA's abundance has decreased or disappeared in all stations except for a one time dramatic increase in station 710 during August 2016 (also see Appendix 9) Additionally, detection is common in offshore stations, hence, the fluctuations in these stations are a bit clearer.



a) Stations near the shoreline



b) middle stations



c) offshore stations

Figure 11. Fluctuations in the Cq mean (eDNA abundance) and temperature per station across the sampling months.

Note: Nieuwpoort stations ——; Oostende stations ——; Zeebrugge stations ——

Chapter 5: Discussion

5.1. *C. hysoscella* (scyphozoa) and its eDNA in the Belgian coast

To date, there is limited knowledge about the distribution of scyphozoans in the North Sea. Most of the available information about this jellyfish group is quite old and only few recent studies are available. Some studies focused on the entire North Sea but provided limited data for the southern part of North Sea (Möller, 1980c; Hay et al., 1990), others studied only the Dutch coast (Verwey, 1942) and the British Isles (Russel, 1970). Researches on scyphozoans in the southern North Sea (Hartlaub, 1894; Künne, 1952; Kopacz, 1994) and the Elbe Estuary (Kühl, 1964; Thiel, 1966) were also obsolete. Some of the most recent studies concerning jellyfish in the North Sea which provided data on scyphozoans include Lynam et al. (2005), Barz and Hirche (2007), De Blauwe (2013), Duliere et al. (2014), Bastian et al. (2014), Vansteenbrugge et al. (2015) and Gambill (2016). Despite these recent studies about scyphozoans in the North Sea, little is known about the scyphozoan jellyfish *C. hysoscella*. *C. hysoscella* occurs mainly in the southern North Sea (Hay et al. 1990). Although this species is one of the most common scyphozoans in the southern North Sea and in the BPNS, it is the least studied species compared to *A. aurita* and *C. lamarckii*. Even the literature does not provide sufficient information about *C. hysoscella*. Hence, there is a need to gather and build information about this species. The need to study this species is more important in locations where the species has been reported to impact the marine environment as well as tourism such as the Belgian coast.

The present study is the first attempt to monitor the presence and the distribution of the compass jellyfish *C. hysoscella* in the BPNS using the eDNA approach. The results obtained from the present study showed that *C. hysoscella* eDNA is present in the BPNS depending on the sampling time and the location in the Belgian coast. The presence of such eDNA can be an indication of the species' presence in the study areas. Additionally, the amount of eDNA present in the study areas may reflect the abundance of the *C. hysoscella* present in the study locations. In this study, *C. hysoscella* eDNA abundance varied depending on the sampling month and the location within the Belgian coast. According to the Marine Life Information Network (MarLIN) (2017), *C. hysoscella* is distributed in British

and Irish coasts. The jellyfish occurs in coastal waters all around the British Isles and prevalent off the south and west coasts of England and Wales. It is also found off the Cumbrian coast, the Isle of Man and north coast of Ireland. The Ocean Biogeographic Information System (OBIS) also recorded the occurrence of *C. hysoscella* in the southern North Sea particularly in the coasts of Belgium, Netherlands and Germany. The jellyfish also occurs in the North Atlantic (France) and South Atlantic (South Africa).

Based on the records of occurrence from OBIS (2017), there are 229 records of *C. hysoscella* occurrence in the United Kingdom since 1960, 167 records in Ireland since 2005, 10 records in Belgium since 1997, 5 records in Germany since 1990 and 2 records each for the Netherlands, France and South Africa. In Belgium, the jellyfish is found to be common in Oostende, Nieuwpoort and in Zeebrugge (OBIS, 2017) and this coincided with the results of the present study wherein *C. hysoscella* eDNA was found frequently in Oostende stations and least in Zeebrugge stations. From 2002 onwards, records on the overall occurrence of *C. hysoscella* tend to fluctuate. However, from 2008 until 2011, there was a consistent increase in the occurrence of the jellyfish. But from 2012 to 2014, the occurrence of the jellyfish dramatically declined (OBIS, 2017). The results of this study showed that the frequency of occurrence of *C. hysoscella* eDNA in the BPNS has decreased from October 2014 to August 2016 (see Table 3). Additionally, a decreasing trend in the concentration of *C. hysoscella* eDNA and even disappearance (non-detection) of the jellyfish' eDNA has been observed in most stations after October 2014 to August 2016 except for stations 710, ZG02 and 780 from which an increase in the eDNA abundance has been recorded (see Table 5). If the trend in the occurrence, distribution and change in abundance of the target eDNA recorded in this monitoring study reflected the actual trend in the existence, patterns of distribution and the actual density of *C. hysoscella*, a decreasing occurrence and number of the jellyfish in the BPNS can be claimed.

The current study frequently detected *C. hysoscella* eDNA in offshore stations of the BPNS and less in the shoreline stations. This result contradicted those of other studies on *C. hysoscella* which showed that the jellyfish species is mostly coastal (Russel, 1970). *C. hysoscella* ephyrae were usually found in shallow areas (e.g., inshore or coastal). The

frequency of occurrence of *C. hysoscella* ephyrae was highest inshore (with a depth of approximately 50 meters) and decreased in depths more than 50 meters (offshore) (Buecher and Gibbons, 1999). However, the definition of shallow and deep study area varies per study location. Considering the southern North Sea to which the study locations were established, the offshore stations (ZG02, 330 and 780) can still be considered shallow considering their depths (see Appendix 1). The BPNS is a temperate shallow shelf. The water depth near the coastal area is usually less than 20 m and this increases to approximately less than 50 meters further off the coast (Emeis et al., 2015; Belgische Staat, 2012; Barz and Hirche, 2007). With a shallow offshore as compared to the other coasts, it is therefore possible for *C. hysoscella* species to occur in the offshore stations and this was confirmed by the presence of its eDNA farther from the coastline. Doyle et al. (2007) has shown that *C. hysoscella* was frequently observed in the shallow Celtic Sea (Transect 1) and absent in the part of the Celtic Sea closer to the St. George's Channel (Transect 2) and in the deeper Irish Sea (Transect 3). Along the first transect nearer to Ireland, *C. hysoscella* was observed in greater density offshore compared to the most coastal section of the transect. Barz and Hirche (2007) examined the horizontal and vertical distribution of scyphozoan jellyfish (which included *C. hysoscella*) in the southern North Sea. Considering the horizontal distribution of the scyphozoans, most of them were found near the shore including *C. hysoscella*. For the vertical distribution, they revealed that *C. hysoscella* medusae were caught deeper stations (15 to 20 m depth). In the current study, the middle and offshore stations established in the BPNS is from 7 to 24 m depth (see Appendix 1), almost close to Barz and Hirche's (2007) station depth where *C. hysoscella* were caught. The results of these cited studies imply that despite being described as coastal species, the distribution of *C. hysoscella* can't just be limited near the shore. They could also be found in slightly deeper regions. Knowing this possibility, it is therefore possible to recover *C. hysoscella* eDNA in deeper regions in the BPNS as what was observed in the current study.

This study demonstrated that the abundance of *C. hysoscella* eDNA in the BPNS somehow varied spatially and temporally although the degree of the observed variations is small (see Figures 9 and 10). The qPCR of the eDNA samples from the BPNS consistently

showed small amount of *C. hysoscella* eDNA when present (see Appendix 7). Based on the average Cq means from the qPCR of the eDNA samples, peaks on the eDNA abundance occurred in October 2014, March 2015, August 2015 and August 2016 (see Table 5). However, the general trend observed in this study was that the eDNA abundance from October 2014 to May 2016 had decreased and was recorded to increase during the last sampling time (August 2016). Although *C. hysoscella* is one of the common gelatinous species in the North Sea, its density compared to the other North Sea gelatinous species is noticeably low. Vansteenbrugge et al. (2015) revealed that the scyphozoans (to which *C. hysoscella* is included) in the BPNS had the lowest total average density (individual per cubic meter) compared with ctenophorans and hydrozoans. Looking at the density of the scyphozoan species, *C. hysoscella* together with *A. aurita* had a density of <0.01 (*C. lamarckii* being the most abundant). The occurrence in low density of *C. hysoscella* in the BPNS might possibly explain why the amount of detected eDNA from this species in the Belgian coast is low. The dilution of the target eDNA, its degradation and transport by current might have contributed more to the low amount of eDNA collected in the sampling stations in the BPNS. But this assumption must be investigated since it was not studied in the present study. Barz and Hirche (2007) previously made a study on the abundance and distribution of the scyphozoan medusae *A. aurita*, *C. lamarrckii*, *C. capillata* and *C. hysoscella* in the southern North Sea in 2004 and 2005. They were able to record the presence of *C. hysoscella* during 2004 only in the months of August and September (summer and autumn) in the Dutch coast. In 2005, *C. hysoscella* was only recorded in the month of July (summer) in the coastal region of Heligoland Island, Germany. A survey of gelatinous plankton within the harbor of the North Sea (part of Dunkirk, France) recorded the occurrence of *C. hysoscella* from April to August (spring to summer) Bastian et al. (2014). Vansteenbrugge et al. (2015) detected the compass jellyfish only during summer and autumn in the BPNS. However, the present study was able to detect compass jellyfish' eDNA across the sampling months. The results of the stated studies imply that there is time variability in sighting or catching *C. hysoscella* medusae. But based on the recently available data, *C. hysoscella* appearance occurs during spring, summer and autumn period. These periods of appearance coincided with the results

obtained from this study which showed that *C. hysoscella* eDNA is at its peak during spring, summer and autumn.

The detailed description of *C. hysoscella*'s life cycle has not been elucidated yet. However, Agassiz (1860) was able to firstly document the life cycle of two scyphozoan species (*A. aurita* and *C. capillata*) known as the Metagenic Life Cycle (MLC) (as cited by Ceh et al., 2015). Agassiz' (1860) MLC model remains the fundamental model for understanding the ecology of scyphozoans and is thought to reflect the extremely seasonal environment of many scyphozoans. Generally, the MLC reflects that when environmental conditions and abundant resources are favorable for growth and reproduction, medusae (motile and pelagic life forms of scyphozoan) is maintained. When resources become limiting and when the environmental conditions downgrade, polyps (sessile and benthic life forms) are maintained and adapted (Boero et al., 2008). According to Agassiz' model (1860) as cited by Ceh et al. (2015), in early spring, scyphozoan ephyrae develop into young medusae which marks the start of the pelagic phase of the scyphozoan life cycle. The young and motile medusae grow into sexually mature medusae through summer. After sexual reproduction, the mature medusae subsequently die (occurs during fall or autumn). The pelagic planulae which were previously produced by the sexually mature medusae sink to the seabed and metamorphoses into sessile polyps (scyphistoma) and marks the start of the benthic phase of the scyphozoan life cycle (starts during fall or autumn). Scyphistomae reproduce asexually through strobililation resulting to the production of cysts (occurs until winter). In early spring, scyphistomae develop into strobilae and releases the ephyrae. These ephyrae then develop into young medusae which again marks the beginning of the pelagic period of the life cycle.

Ceh et el. (2015) revisited the metagenesis in scyphozoan jellyfish. They studied the population dynamics of *Chrysaora plocamia* for three years (2010-2013). They found out *C. plocamia* traits that were difficult to interpret in the light of the Agassiz' (1860) MLC model. *C. plocamia* medusae demonstrated uneven seasonal pattern. Medusae were observed from early to late summer (November-February). However, the months where *C. plocamia* medusae were detected in all the years were limited to November, December and January

(autumn and winter). Additionally, medusae were also recorded as early as October (autumn), as late as March (spring), in April (spring) and even in winter months (June and July). In 2011/2012, the medusae re-appeared after the summer season (autumn and winter). Upon reviewing a wide range of literature about scyphozoans which reported diversions with the MLC model and after analyzing the results of their study, they had suggested additions to the current MLC model. According to Ceh et el. (2015), scyphozoa medusae in surface waters in late spring are large and sexually mature while the medusae found in the later season (summer) tend to be smaller in size. In late summer, medium-sized medusae sink to the sea bed and spend the autumn and winter periods near the deep benthos and ascend as large and sexually mature medusae to surface waters in the late spring. During summer period, polyps produce a mobile planuloid through external/internal gemmation, bypassing the medusa-stage. The planula-larvae produced during this season can directly develop into ephyrae by-passing the medusa-stage. Strobilation during the summer period supplies new and smaller medusae at summer time. These suggestions imply that scyphozoan medusae overwinter. This means that not all the medusae die during the medusa-season (spring and summer). Some sink to deeper water, overwinter and may re-appear to surface waters as large and sexually mature medusae during spring. Additionally, Ceh et el. (2015) implied that polyps produce ephyrae throughout spring and summer and that polyp-and medusa generations are not temporally and spatially separated.

It is difficult to conclude which life cycle perfectly and truly fits *C. hysoscella*'s especially that Ceh et al. (2015) did not include data on *C. hysoscella* in making their suggestions to the scyphozoan life cycle. Additionally, the present study used eDNA approach in detecting the compass jellyfish and not based on sightings or catches. Therefore, it is impossible to see the variation in the size of the *C. hysoscella* medusae present in the BNPS throughout the sampling periods. However, aside from basing their conclusions from *C. plocamia*, they also used literature data from *Chrysaora melanaster*. Knowing that *C. hysoscella* belongs to the same genus as *C. plocamia* and *C. melanaster*, the three species are therefore closely linked to each other and there is a possibility that their

life cycle would have greater resemblance. It is therefore safe to assume that *C. hysoscella* life cycle may follow closely the proposal of Ceh et al. (2015) than that of Agassiz' proposal as the latter was based on a more distant relatives of *C. hysoscella*. But this claim must be verified in *C. hysoscella* future studies. In the present study, water samples were collected on surface waters (3 m from the water surface) and eDNA's were extracted from the collected water and then analyzed for the presence of *C. hysoscella* eDNA. Following the MLC model of Agassiz (1860), the detected *C. hysoscella* eDNA could have been from *C. hysoscella* ephyrae and medusae since these are both pelagic forms and therefore found in the surface waters. With Ceh et al.'s (2015) proposal, the collected eDNAs might have been derived from the polyps, ephyrae and medusae. There were detections of *C. hysoscella* eDNA across the sampling months in the present study with August 2015 and August 2016 (summer periods) consistently revealing higher eDNA abundance. This is expected as during this period, actively sexually reproducing *C. hysoscella* medusae could be present in the water surface. However, considering the MLC model, it is possible that the collected eDNAs were derived from remnants of dead medusae or could be from the ephyrae, young medusae or remnants of dead larger medusae if the new proposal is to be considered. Although not consistent, greater amount of eDNA during the months of October 2014 (autumn) and March 2015 (spring) was recorded (even higher that the amount of eDNA during August 2015). One possible explanation for this is the nutrient availability in the water column during these months. Spring is characterized by a strong phytoplankton bloom (diatoms and flagellates) followed by a zooplankton bloom (Daro *et al.,* 2006; Van Ginderdeuren *et al.,* 2014). Copepods might increase during this zooplankton bloom. Copepods are known as food sources for jellyfish. Their increase may lead to the simultaneous increase of jellyfish. Finally, in autumn, a smaller secondary bloom occurs, as increased mixing breaks down the thermocline and nutrients are released again (Hay *et al.,* 2011). Changes in the hydrodynamics in the North Sea such as warming and changes in wind-driven water currents and vertical mixing do not only shift the timing for spring phytoplankton bloom and the composition of zooplankton (Graham et al., 2001; Hay et al.,

1990; Lynam et al., 2005a, 2004) but also significantly affect the development, survival and, hence population size of jellyfish (Purcell, 2005).

Whether jellyfish blooms occur globally or not is still an issue in the field of marine research (Gibbons and Richardsons, 2013). There are inconsistencies on the findings concerning jellyfish blooms. A research outcome suggests that there is no increase (Condon et al., 2012) and another suggests that global increase in jellyfish is happening (Duarte et al., 2013). Contributing to these inconsistencies and inconclusive findings is the fact that reliable jellyfish data is lacking (Purcell, 2009; Brotz et al., 2012; Condon et al., 2012). Additionally, the language to describe the increase in the jellyfish population is another problem which can contribute to misconception and misinterpretation of jellyfish data (Lucas and Dawson, 2014). It must be noted that the commonly used terms associated with jellyfish population increase such as accumulation, mass occurrence, aggregation, blooms, outbreak and swarm technically mean differently. Consistency of term usage is important for understanding the causes and consequences of blooms (Lucas and Dawson, 2014). The observed increase in the number of *C. hysoscella* in several locations in the Belgian coast on August 2013 (De Blauwe, 2013) was described to be a jellyfish swarm by Duliere et al. (2014). A numerical model based on the Lagrangian particle approach was used to backtrack the origin of the *C. hysoscella* swarms in August 2013. The model predicted that the observed *C. hysoscella* in the Belgian coast originated from an offshore area within the Belgian waters (Duliere et al., 2014). The same approach was used to backtrack the origin of the stranded jellyfish swarms in the Belgian coast (Oostende) on May 2013. The model showed that the stranded jellyfish originated from the UK coast (Duliere et al., 2014). Knowing that *C. hysoscella* occurs more frequently in the whole United Kingdom and that these species may follow the water current movement, it is possible that most of the jellyfish found in the BPNS could somehow originated from the UK coast. The evident surface current in the southern North Sea (Vlaeminck et al., 1989; Lacroix et al., 2004) can possibly transport jellyfish from UK to the Belgian coast. Water current passing through the English Channel to the Belgian coast and the water current on the eastern coast of England moving south-east could bring jellyfish to the BPNS. The physical boundaries of the North

Sea serve as aggregation sites (Graham et al., 2001; Hay et al., 1990; Lynam et al., 2005a, 2004), since jellyfish as part of the plankton community are not able to actively swim against currents (Gambill, 2015). Their distribution seems to be forced by the effects of density driven currents and the presence of frontal zones. High abundances of scyphomedusae are expected in stratified waters, bordering estuaries (Doyle et al., 2007; Nielsen et al., 1997) and pycnoclines (Graham et al., 2001).

Higher oceanic temperature may lead to the proliferation of many populations of gelatinous species since sea temperature can influence jellyfish life cycles and their reproductive performance and output (Purcell et al., 2007; Boero et al., 2002). Rising marine temperature changes the timing and duration of the pelagic stages of jellyfish reproduction (Purcell et al., 2007; Ceh et el., 2015). Moreover, warming of the ocean may also affect and shift the population distributions poleward of some species, as appears to be occurring for the ctenophore *Mnemiopsis leidyi* (Faase and Bayha, 2006; Hansson, 2006). However, warming temperature does not always lead to increase in jellyfish number since it can be lethal in some species (Dawson et al., 2001). *C. melanaster* was found to increase in the Bering Sea during the warming from 1976 to 1977, increasing most dramatically in the 1990s, but then decreasing with further warming since 2000 (Brodeur et al. in press). The present study investigated the correlation between the temperature of the sampling stations recorded during sampling and the amount of eDNA present in the sample (as represented by the Cq values generated by the qPCR). The result suggests that temperature is not correlated with the amount of eDNA present in the BPNS (p value = 0.4254). This absence of correlation is expected and evident in Figures 9 and 11. Considering the general claim that warmer temperature leads to higher number of jellyfish, it can somehow be expected that at warmer sampling months at least we should have detected more *C. hysoscella* eDNA (e.g., lower Cq mean). Although lower Cq means were recorded for warmer months (October 2014, August 2015, August 2016), there were warmer sampling months wherein higher Cq means were recorded (October 2015, May 2015, May 2016) and colder months (March 2015, January 2016, March 2016) where Cq means were lower than in warmer months. Buecher and Gibbons (2000) demonstrated that *C. hysoscella* ephyrae

are eurythermal however, their frequency of occurrence is higher in lower temperature. Interestingly, there is a possibility that higher amount of eDNA was detected in colder months due to the absence of light or presence of low light intensity during these periods than in warmer months. Hence, less degradation of eDNA occurred in colder months than in warmers months. Further investigations relating the amount of *C. hysoscella* eDNA in the BPNS with temperature is required to settle the inconsistencies recorded in the present study.

5.2. eDNA approach as a tool in monitoring *C. hysoscella* in the BPNS

Species detection using eDNA can potentially contribute to our understanding on the ecology of aquatic species. This monitoring approach has been claimed by many as more effective technique in detecting and monitoring species than the conventional detection methods (Thomsen et al., 2012a, Dejean et al., 2011; Keskin, 2014;). Detection and quantification of species-specific eDNA can possibly eliminate the downsides of traditional sampling methods. eDNA sampling requires small amounts of water (e.g., 1 L or less) (Thomsen et al., 2012a; Takahara et al., 2013) and species-specific primers and probes for target eDNA quantification through qPCR. With this, the eDNA approach has species-specific sensitivity and obviously requires less time for field sampling compared to the conventional surveying methods. Therefore, eDNA technique can be a promising tool for marine species detection and for monitoring species dispersal and patterns of distribution (Takahara et al., 2013). Looking for eDNA rather than direct sampling of organisms can reduce impacts on sensitive species and increase the power of field surveys for rare and elusive species (Goldberg et al., 2016). With its potential, determining how well eDNA can serve as a proxy for directly observing organisms must be intensively explored to reliably apply the technique in monitoring all types of ecosystem.

This study showed that the presence or absence of *C. hysoscella* in the BPNS can be determined using the eDNA approach. The qPCR data of the eDNA samples from the BPNS showed that *C. hysoscella* eDNA abundance somehow exhibited spatial and temporal variation. Hence, the eDNA approach provided an insight on the locations and the sampling months *C. hysoscella* appeared in the Belgian coast. Additionally, the differences in the Cq

means (or abundances) of the eDNA samples provided a snapshot on the abundance of *C. hysoscella* in the sampling stations across the different sampling time. This monitoring technique showed the patterns of distribution of the compass jellyfish in the Belgian coast. The monitoring approach used in this study interestingly showed that from 2014 to 2016, the frequency of occurrence of *C. hysoscella* in the Belgian coast had decreased. Since monitoring and detection of *C. hysoscella* using eDNA is the first to be conducted based on the absence of similar studies in the literature, it is difficult to validate the results obtained from this eDNA study. However, using the limited information about the surveys, occurrences, sightings, catches, temporal and spatial distributions of *C. hysoscella* in the North Sea and in other locations previously presented in this paper, the time and space distribution as well as the abundance of the compass jellyfish as predicted by the eDNA data of this study reflected the findings obtained from actual observations of the jellyfish. However, it is important to note that the eDNA results obtained in this study were interpreted without considering and investigating the possible impacts of eDNA dilution, degradation and transport by surface water movement. These could be interesting topics that need to be explored to further develop the method in monitoring species from marine environment.

The sensitivity of eDNA technique demands a sensitive awareness and consideration to quality assurance and quality control protocols. Also, multiple factors are need to be considered when interpreting eDNA data and results (Goldberg et al., 2016). In eDNA study like this, following the protocols is very important. The extraction procedure needs to be performed accurately. The eDNA extraction method used in this study was considered efficient as for all extracted samples, NanoDrop measurements showed extracted eDNA. However, to have a good quality of eDNA extracts, care should be considered during extraction. Simple things like working on eDNA filters must be dealt with dedication. The forceps and the scalpel used to cut filters must be always decontaminated after and before using it to another eDNA filter. This reduces the chance of cross-contamination. Additionally, the collection of the filtered material into the Effendorf tubes must be maximized. Even those adhered into the forceps or knife must be collected into the tubes

to not miss the filtered eDNA from the water samples. Pipetting of reagents, the time of exposing the eDNA extract with these reagents must be accurate. Pipetting the supernatant from eDNA extract solution must be done with utmost care and dedication. This is very critical for determining the purity of the eDNA extract. When cleaning the eDNA pellets with the solvent, pipetting should be done correctly as to not remove the pellets from the tube and be discarded with the solvent. This obviously can affect the result of the detection of the target eDNA.

Not all of the eDNA extracts used in this study had the ideal 260/280 and 260/230 ratios. The NanoDrop also gave negative ratios. These negative ratios are usually due to a blank measurement that was made either using a solution with more absorbance than the sample buffer or on a dirty pedestal (ThermoScientific, 2017). However, the blank solution used in the NanoDrop and the buffer solution used in the sample were the same and the pedestals were always cleaned using the appropriate wiping paper. Even after re-blanking and re-cleaning the pedestal, negative ratios still appeared. It is strongly recommended that eDNA samples that did not meet the standards for both 260/280 and 260/230 ratios be not included in the qPCR analysis to reduce issues on qPCR inhibition in the outcomes. This means multiple eDNA extracts from one sampling location must be prepared. However, it is important to mention that most of those extracts that did not reach the ideal ratios, still showed amplification in the qPCR (see Table 5). The qPCR results showed false positive results in some qPCR plates. A test was done to trace possible contamination during the qPCR step (see Table 4). The result of this test revealed that the mastermix used in the qPCR was not contaminated with *C. hysoscella* eDNA since it did not show any amplified products (see Figure 8). However, amplified products were present in the NTCs which only contained primers. This suggests that when primers were added in the PCR/qPCR reaction solution even without any template DNA, amplified products can be produced. This supports the claim that primer dimerization may have caused the appearance of the amplified products in the NTCs (e.g., resulting to false positive outcomes) during the qPCR. Primer-dimer is a product of duplex formation between two primers. Primers bind to each other and elongated by DNA polymerase. Formation of primer-dimers not only decreases the

concentration of primers in the reaction mix but also initiates the formation of nonspecific DNA products (Das et al., 1999). Primer dimerization can be avoided by adding a sequence of nucleotides at the 5'-ends (inactive side) of the primers (Brownie et al., 1998). Primer-dimers can also be reduced by optimizing the PCR/qPCR reaction conditions such as carrying out the annealing of the primer and synthesis at higher temperature (Wu et al. 1990, Rychlik et al. 1990). Roche® also has manuals which give suggestions on how to reduce primer-dimer formation. To opmitize the eDNA protocol for *C. hysoscella*, these considerations must be taken into account. Focus must be given to the currently designed *C. hysoscella* primers and to the reaction conditions during qPCR/PCR to improve the currently developed protocol.

eDNA studies in marine ecosystem comes with several challenges. eDNA in marine system is diluted due to a larger water-volume to biomass ratio, easily and rapidly dispersed due to sea-currents and wave action and less efficiently extracted from the water column due to saline condition (Thomsen et a., 2012a) due to these, it is not evident whether using eDNA technique can represent the distribution and biomass of marine organisms since various environmental factors are expected to affect eDNA distribution (Yamamoto et al., 2016). Goldberg et al. (2016) made an interesting review paper pointing out critical considerations for the application of eDNA to detect aquatic species. The authors also presented issues for eDNA researches that must addressed to improve the application of eDNA method and non-biased result interpretation. Such issues include: inference across space and time, inferring presence versus viable populations and confounding sources of eDNA. The preliminary data obtained from this study suggests that eDNA can potentially detect the occurrence of *C. hysoscella* by sampling its eDNA from surface water. This implies that the method can be developed further to assess or monitor jellyfish species in marine ecosystem especially if abundance, temporal and spatial variations of jellyfish are to be investigated. Just recently, the first study on jellyfish (*Chrysaora pacifica*) distribution using eDNA has been published (Minamoto et al., 2017). Spatial and temporal distribution visual surveys of *C. pacifica* were conducted at Maizuru Bay, Kyoto which were coupled with collecting surface water and sea floor water samples for *C. pacifica* eDNA detection and

quantification. Interestingly, after eDNA data analysis, results reveal that *C. pacifica* eDNA distribution and abundance reflected the spatial and temporal distribution of jellyfish inferred by visual surveys. Additionally, their results suggested that the concentration in the bay was significantly higher on the sea floor than on the surface. The study of Minamoto et al. (2017) shows that eDNA approach can be effectively used to assess the spatial and temporal distribution of jellyfish. Therefore, with more intensive research and optimization of protocols, the distribution and abundance of *C. hysoscella* and other jellyfish in the North Sea can be effectively monitored by sampling their eDNA either from water surface or from the sea floor/bottom water.

Chapter 6: Conclusions and recommendations

The results obtained from this study suggest that *C. hysoscella* in the BPNS can be detected by sampling its eDNA. *C. hysoscella* eDNA in the BPNS are commonly found offshore and exhibited a rather less evident temporal and spatial distribution variations. The compass jellyfish' eDNA frequently occurs in Oostende, Nieuwpoort and least in Zeebrugge. Although the occurrence of the *C. hysoscella* eDNA has decreased from 2014 to 2016, peaks in eDNA are observed in October 2014, March 2015 and August 2015 and 2016. The jellyfish' eDNA abundance did not show any correlation with the surface water temperature. These results imply that eDNA approach can be used to detect *C. hysoscella* and other jellyfish in the North Sea to study their distribution and abundance. This monitoring technique is important in predicting possible blooms of the North Sea jellyfish which in turn is necessary to reduce the negative impacts of such blooms.

To further develop eDNA approach in monitoring *C. hysoscella* and other North Sea jellyfish, the following are recommended:

1. Observe utmost precautions when doing eDNA experiment and optimize the PCR/qPCR part of the eDNA protocol. Explore the best PCR/qPCR condition for *C. hysoscella* and improve the primers designed for this species.
2. Conduct a tank experiment to explore the degradation of *C. hysoscella* eDNA.
3. To validate the applicability of eDNA in monitoring *C. hysoscella* this approach must be coupled with spatial and temporal surveys to compare outcomes of the two monitoring methods.
4. Analyze the *C. hysoscella* eDNA distribution for all months in a year and include bottom waters to see clearer patterns and distribution and relate these to some abiotic factors.
5. Use and verify eDNA approach with the most common and abundant jellyfish in the North Sea like *Cyanea lamarckii* or *Mnemiopsis leidyi*.

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Appendices

Appendix 1. The nine study locations established in the Belgian part of the North Sea (BPNS) with their corresponding coordinates and depths.

Station	Latitude	Longitude	Depth (m)
120	51°11.145	2°42.1	10
215	51°16.648	2°36.797	24
ZG02	51°19.962	2°30.052	13
130	51°16.06	2°54.06	10
230	51°18.473	2°50.916	12
330	51°26.024	2°48.482	21
700	51°22.563	3°13.183	10
710	51°26.413	3°8.252	7
780	51°28.292	3°3.523	19

Appendix 2. Protocol on CTAB-based extraction of high-molecular weight DNA from eDNA filters. (Responsible: Jana Asselman)

1. Goal

For some molecular applications, such as sequencing and Copy Number Variation Analysis, high molecular weight DNA is needed. This protocol describes a method for obtaining such a high molecular weight DNA from *Daphnia* sp. The protocol is based on the use of a cetyltrimethylammonium bromide (CTAB)-based extraction buffer. Nucleic acids can be selectively precipitated with the cationic CTAB. RNA and DNA are soluble in CTAB and 0.7 M NaCl, but precipitate when the salt is reduced below 0.4 M.

Extraction of high quality and high molecular weight DNA is recommended to use in downstream applications such as qPCR (CNV etc.)

This protocol is time-consuming, but it is highly effective. It is based on a protocol obtained from Prof. Joseph Shaw, Indiana University, Bloomington, Indiana, USA, which in turn was a modification of the Lynch Lab protocol (Abe Tucker, 4/2009).

2. Equipment and Reagents

2.1 Cetyltrimethylammonium bromide (CTAB) (Sigma-Aldrich, H5882)

2.2 β-mercaptoethanol (Sigma-Aldrich, M6250)

2.3 Sodiumchloride (NaCl) (Merck, 27810.295)

2.4 Tris-base (Sigma, T1503)

2.5 HCl (Prolabo, 20252.290)

2.6 Na_2EDTA (Fluka, 03685)

2.7 Ultrapure, DNase and RNase-free water (Teknova)

2.8 Autoclaved Pestles

2.9 Chloroform (Sigma, C2432)

2.10 Iso-amylalcohol (VWR, 20796.298)

2.11 Phenol-chloroform-isoamyl alcohol 25:24:1 (v/v) (Ambion)

2.12 1,5 mL autoclaved Eppendorf tubes

2.13 RNase A (Qiagen, 19101)

2.14 70% (v/v) EtOH (Merck, 1.08543)

2.15 Sodium acetate (Merck, 6268)

2.16 Microcentrifuge with cooling

2.17 Warm water bath

2.18 Pipettes

2.19 Barriertips for pipettes

2.20 Ice

2.21 Paper

2.22 Vortex

2.23 Nitril gloves (VWR)

2.24 Styrofoam box

3. Solutions and preparations

Note: It is important to use RNAse, DNAse and nuclease-free pipette barriertips when preparing solutions.

Note: With the described quantities one can extract DNA from about 350 samples.

Note: All solutions containing CTAB, β-mercaptoethanol, chloroform, phenol, isopropanol should be discarded in the appropriate waste container!

Note: Some components are toxic or carcinogenic, take the necessary safety precautions (fume hood, gloves) as recommended by your tutor/advisor.

3.1 Solutions

3.1.1 1M Tris-HCl

1M Tris pH 8.0 is generally available in a pre-made solution within the lab.

If not, you can make Tris-HCL as follows: to make 100mL 1M Tris-HCl buffer pH 8.0, add 12.11g Tris to about 50mL MilliQ H_2O. Bring to pH 8.0 with HCl and bring volume up to 100mL with MilliQ H_2O. This solution can be stored at 4°C for up to 6 months.

3.1.2 0.5M Na_2EDTA

0.5M EDTA is generally available in a pre-made solution within the lab.

If not, you can make 0.5M EDTA as follows: to make 100mL 0.5M EDTA pH 8.0, add 18.61g $Na_2EDTA.2H_2O$ to about 50mL autoclaved MilliQ H_2O. Adjust the pH to 8.0 with NaOH solution (The reason to adjust the pH is that the EDTA will not dissolve until the pH is about

8). Bring volume up to 100mL with MilliQ H_2O. This solution can be stored at 4°C for up to 6 months.

3.1.3 CTAB extraction buffer

For 50mL CTAB extraction buffer, add 4.09g NaCl to 40 mL ultrapure H_2O. Then add 5mL 1M Tris-HCl pH 8.0, 2mL 0.5M Na2EDTA pH 8.0 and 1g CTAB. Finally, **after adding all the previous components**, add 100µL 14.3M β-mercaptoethanol. Heat at 65°C. Make **fresh** extraction buffer every time. This solution can be stored for maximum 3 days at 4°C.

3.1.4 3M Sodium Acetate

3M Sodium Acetate is generally available in a pre-made solution within the lab.

If not, you can make it as follows: for 50mL 3M NaAc, dissolve 12.3g NaAc in about 25mL MilliQ H_2O. Add volume up to 50mL with ultrapure H_2O. This solution can be stored at 4°C for up to 6 months.

3.2 Preparations

The following preparations need to be made before starting the DNA extraction:

- Turn on a warm water bath at 65°C
- Prepare the phenol:chloroform:isoamyalcohol (see instructions with bottle)
- Prepare the chloroform:isoamylalcohol solution 24:1 (e.g. 48mL Chloroform + 2mL Isoamyl alcohol for ~50 samples)

4. DNA extraction

Note: It is important to use RNAse, DNAse and nuclease-free pipette barriertips. Clean used materials beforehand and afterwards with 70% EtOH.

1. Option 1 – tissue. Add 300µL of warm CTAB extraction buffer (65°C) immediately.
   Option 2 – filter: Cut filter into smaller pieces with a clean dissection knife (soaked in 10% bleach for 10 min and rinsed with deionized water). Add 300µL of warm CTAB extraction buffer (65°C) to the filter samples.
2. Option 1 - Grind tissue in warm CTAB extraction buffer using pestle. Twist, plunge, mash and grind for a good 30 seconds or more, being sure not to create to many bubbles by pulling the pestle in and out of the liquid.

Option 2 - Add beads to the filter sample and vortex vigorously for minimum 10 minutes to disrupt the DNA from the filter.

3. Quick vortex to mix. Incubate at 65°C (water bath) for 1 hour. Vortex every 20 minutes during this 1-hour incubation.
4. Remove tubes from 65°C water bath and in the fume hood, add 300 µL phenol:chloroform:IAA 25:24:1 (v:v:v). When pipetting the phenol solution, avoid the top aqueous layer and only pipet from the bottom layer. This solution is very sticky and requires slow aspiration.
5. Vortex vigorously to mix and then spin tubes at 15000 rcf for 15-20 minutes in a cooled microfuge (4°C).
6. Leaving debris and organic layer behind, carefully pipette out the supernatant into a clean autoclaved 1.5mL tube.
7. Add 1 µL of RNase A to a final concentration of 10µg/mL (i.e. 5µL of 1000µg/mL RNase A). Incubate on benchtop 20 min.
8. Prepare Phase Lock Gel (PLG) tubes by centrifuging them for 30s at high speed on room temperature.
9. Add 500µL of 24:1 Chloroform: Isoamyl alcohol (C:I) (v/v) and mix vigorously. Transfer everything into the PLG tube. Centrifuge at 15000 rcf for 15 minutes at room temperature (on average 21°C).

   Note: Use glass instead of plastic to prepare the 24:1 Chloroform: Isoamyl alcohol solution!
10. Transfer aqueous layer (supernatant) to clean 1.5mL tube avoiding scum at the interface.
11. Add 27µL of Na Acetate (3M). Then add 500µL of 2-propanol. Invert to mix. Put to 20 °C for 10 mins.
12. Spin samples at 4°C, 10000 rcf for 15 minutes to pellet.
13. Check for pellet. Then gently pipet off the 2-propanol.
14. Add 500µL 70% EtOH to wash pellet.
15. Spin samples at 4°C, 10000 rcf for 15. Then gently pipet off the 70% EtOH.

16. Add 500µL 70% EtOH to wash pellet.
17. Spin samples at 4°C, 10000 rcf for 15. Then gently pipet off the 70% EtOH.
18. Allow pellet to dry (air-dry or vacufuge)
19. Resuspend pellet in 50 µL 10 mM Tris, pH 8.0 or with 30µL of 1X TE Buffer
20. Store sample at - 20 °C

5. Quality control

Check purity of your extracted DNA on a Nanodrop (see protocol "Measuring DNA-RNA purity and concentration on the NanoDrop"). Values should be between 1.7 and 2.0 for 260/280 ratio and greater than 2.0 for 260/230 ratio.

Integrity of your DNA extract should be checked by gel electrophoresis. The gel image should show high molecular weight DNA (i.e. single band above the highest marker) with no other bands or smear present on gel.

Appendix 3. Protocol on primer and probe design for qPCR. (Responsible: Tara Grosemans)

1. Goal

The goal is to make good primers and probe that can be used in quantitative PCR (qPCR) experiments. Primers and probe are tested for specificity, hairpins, self-dimers and melting temperature.

2. Method

1. Go to Genbank (http://www.ncbi.nlm.nih.gov/) and choose the sequence for which you want to design primers and a probe.
2. If you have the desired sequence, go to http://eu.idtdna.com/PrimerQuest/Home/Index
3. You can paste the sequence in the sequence box and give the sequence a name.
4. To adjust the parameters, click on Show Custom Design Parameters.
5. Click on qPCR (2 primers + probe). Now you get different assays with their specifications. A good primer has a length around 20 bases, a melting\ temperature (Tm) between 60 and 64°C, which has a max. difference of 2°C between the two primers, and a GC content between 35-65%. The Tm for the probe should ideally be 6-8°C higher than the primers.
6. To test for hairpins and self-dimers, stand on the desired primer or probe row, hairpin and blast will now appear. Click on hairpin. Look in the result table at the free Gibbs energy (ΔG), which should be weaker (more positive) than -9.0 kcal/mole. Look also at the Tm of the secondary structure. The Tm should be much lower than the Tm of the primers.
7. Primers and probe should be specific for the species you are interested in. You can test the primer pair specificity by blasting it. Go to http://www.ncbi.nlm.nih.gov/tools/primer-blast/ and paste the primer sequences in the boxes. Put database on nr and do not specify for the organism (delete *Homo sapiens*), so that you get all possible bindings of the primers to organisms. Click Get Primers.

8. Now you get an output with all sequences (of all organisms in the database) on which the primer can bind to. The returned results consist often of a long list of all possible bindings. The first results will ideally show no mismatches with the primers, meaning that on these species the primer can bind to. These top results should be sequences of the species and the gene you selected. The further you go down the list, the more mismatches will appear and the less likely it is that the primers will work with these species.
9. Blast also the probe against the ncbi database. Stand on the probe row and click on blast. Now you are directed to the ncbi blast webpage. Settings are on blastn (blasting for nucleotides) and click blast. Now you get a table with the sequences containing a match with your probe sequence. The best results are in the top rows. First in the table is the name (description), next you see max score, total score, query cover, E value and identity. You have to look at the latter three columns. The query cover indicates if all nucleotides of your probe sequence are covered in the matching sequences. The E-value stands for the expected number of chance alignments; the smaller the E-value, the better the match. Last is the identity (%), which indicates if mismatches occur between the probe sequence and the output sequences.
10. Keep in mind that these are all short sequences. The longer a sequence the more specific it can be. The combination of primers and probe makes sure that the specificity is high.
11. When the primers and probe are species-specific, don't form hairpins and self-dimers and have the same Tm, it is a good assay for further qPCR experiments.

References:

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Appendix 4. Protocol on PCR for eDNA. (Responsible: Tara Grosemans)

1. Goal

The polymerase chain reaction (PCR) can be used to amplify the DNA with species-specific or general primers to obtain the desired sequence. For more information on designing primers, go to the primer protocol. This PCR protocol is based on the paper of Thomsen *et al.* (2012) for environmental DNA (eDNA).

2. Equipment

2.2 Molecular Grade H_2O (Nuclease free water)

2.3 Primers (lyophilized)

2.4 1.5 mL autoclaved Eppendorf tubes

2.5 0.2 mL Eppendorf PCR tubes

2.6 TaqMan Environmental Master Mix 2.0 (Life Technologies)

2.7 Extracted DNA

Available protocols: CTAB-based high molecular weight DNA extraction from *Daphnia*, DNA extraction with Epicentre kit from fish (Actinopterygii)

2.8 C1000^{TM} Thermal Cycler (Bio Rad, Room B01.45/lab 8, inventory number 554)

2.9 Galaxy MiniStar VWR (Room B01.45/lab 8, inventory number 553)

2.10 Pipettes (0.5-2 µl and 2-20 µl)

2.11 Gloves (VWR)

2.12 Ice block

Preparation

Re-suspending primers

Primers are usually received when they are frozen dry (lyophilized). The first thing to do is to spin down primer tubes before opening to make sure that the pellet is at the bottom of the tube. Make a master stock solution of the primers by adding Molecular Grade H_2O (10 times the number of nmol of primer).

100 µM = X nmoles primer + (X × 10 µl of molecular grade H_2O)

Prepare a working stock solution of 10 µM

Now we want to make a working stock of our primer stock. This working stock will be used in further experiments. If contamination should happen, a new working stock can be made of the primer stock. Also, the primer stock does not need to be thawed for each reaction and thus will not be affected by repeated thawing-freezing cycles. So, it is important that the working stock solution is not too large, but also not too small. Ideally, we make a working stock of 100 µl (this is often enough for 100 reactions (1 µl per reaction)). If you plan to do more or less reactions, you can adjust this volume appropriately.

Mix the primer stock very well before making a 1/10 dilution working solution from it.

Take 10 µl of stock solution and add 90 µl molecular grade water. This is the solution that will be used to make a reaction volume for the PCR protocol.

Validation of primers on gel

It is always recommended to run your primer stocks on a gel to make sure there is actual primer in it. Otherwise you need to contact the company immediately.

3. Methodology

- Turn on the C1000^{TM} Thermal Cycler and click run. The machine will give you an option to choose from the saved protocols or to create a new PCR protocol, depending on the PCR conditions you need.
- Choose Thomsen protocol and click run with the following thermal conditions;
  95^{o}C for 7 minutes;
  50 cycles of 94^{o}C for 30 seconds, 50^{o}C for 30 seconds, 72^{o}C for 20 seconds;
  72^{o}C for 5 minutes;
  4^{o}C on hold forever
- Select ‘ok’ (by pressing F1) and the machine will start heating up.
- Thaw the primer work stocks and DNA on ice on time. Do not vortex! Mix by pipetting carefully up and down. Make a 25 µl reaction volume in the Eppendorf PCR tubes by adding the following:
  - 10 µl of molecular grade water
  - 11 µl of TaqMan Environmental master mix

    - 1 µl of forward primer (10µM)
    - 1 µl of reverse primer (10µM)
    - 2 µl of DNA extract of water or tissue samples (concentration ranges between 17 and 2400 ng/µL, optimisation can be needed dependent on the primer-DNA sample combination of the species).
- When the reaction volume is ready, open the PCR machine and place the sample in. Tighten the knob just enough to keep the tubes from opening or being squashed.
- Select 'skip step' (by pressing F3) in order to go to the next step (by pressing F1). Otherwise it stays at 95°C forever. The DNA sample will be amplified following the Thomsen procedure mentioned above.
- The process will take about 2 hours to finish and when all is done the sample will be cooled and kept at 4^{o}C forever. Press F2 to Stop the process, remove the sample(s) from the machine and switch it off. Keep the samples on ice while proceeding to annex 1 or store them at -20 ^{o}C.
- Next the PCR products can be evaluated by gel electrophoresis. See Annex 1 and the Gel Electrophoresis protocol.

References

Thomsen, P. F., *et al*. (2012). Detection of a Diverse Marine Fish Fauna Using Environmental DNA from Seawater Samples. PLoS ONE 7(8): e41732. doi:10.1371/journal.pone.0041732

Appendix 5. Protocol for DNA gel-electrophoresis. (Responsible: Dieter De Coninck)

1. Goal

DNA gel electrophoresis can be used to check the integrity of your extracted DNA.

Note: It is strongly advised to contact a person who ran a gel before to get you informed about all precautions you need to take into account!

2. Equipment

2.1 Agarose (Merck, 162.0125, No. 172)

2.2 EDTA (Fluka, 101135224, No. 36)

2.3 Tris base (Sigma, T1503-1kg, No. 1)

2.4 Boric acid (Sigma, 1.00165.0500, No. 13)

2.5 Deionized water (room B01.45/lab 8)

2.6 70% denaturated ethanol "Disolol" (ChemLab, CL00.1807.5000, No. BK52)

2.7 Nitrile gloves (VWR)

2.8 Filter tips

2.9 Bio-rad MiniSub Cell GT buffer tank with gel tray and fitting comb for 15 slots (room B01.45/lab 8, red delineated zone)

2.10 Bio-rad PowerPac 300 power source (room B01.45/lab 8, inventory number 464)

2.11 Microwave (room B01.45/lab 8, inventory number 487)

2.12 Eppendorf Reference micropipette (red delineated zone in lab 8)

2.13 Micropipettes (lab 8)

2.14 Bio-rad Gel Doc 2000 (inventory number 156) and CCD-camera (inventory number 156A)

2.15 Computer with Quantity One Software (inventory numbers 156B and 156C)

2.16 GeneRuler 1kb Plus DNA Ladder (Fermentas, SM1334, No. -20|50)

2.17 DNA Loading dye (comes with DNA ladder)

2.18 GelRed coloring dye (rack red delineated zone under fume hood lab 8).
CARCINOGENIC, WEAR APPROPRIATE PROTECTION (Long-sleeved labcoat and nitril gloves)!!!

2.19 PCR 0.2 mL strip tubes with attached strip caps (lab 8) (VWR, 82006-606)

2.20 Microspin VWR Galaxy Ministar (inventory number 553)

3. Preparations

3.1 **Prepare a Stock Solution of 0.5 M EDTA**

An EDTA (ethylenediamine tetraacetic acid) solution is prepared ahead of time. EDTA will not go completely into solution until the pH is adjusted to about 8.0. For a 50 mL stock solution of 0.5 M EDTA, weigh out 9.31 g EDTA disodium salt. Dissolve in 40 mL deionized water and adjust the pH with NaOH. Top up the solution to a final volume of 50 mL.

3.2. **Prepare a Stock Solution of 5x TBE**

Make a concentrated (5x) stock solution of TBE by weighing 54 g Tris base and 27.5 g boric acid and dissolving both in approximately 900 mL deionized water. Add 20 mL of 0.5 M EDTA (pH 8.0) and adjust the solution to a final volume of 1 L. This solution can be stored at room temperature but a precipitate will form in older solutions. Store the buffer in a glass Duran bottle and discard if a precipitate has formed.

3.3 **Prepare a Working Solution of 1xTBE**

For agarose gel electrophoresis, TBE can be used at a concentraion of 1x (1:5 dilution of the concentrated stock). Dilute the stock solution by 5x in deionized water.

3.4 Thaw samples, DNA ladder and loading buffer on ice.

4. Methods

Note: It is strongly advised to contact a person who ran a gel before to get you informed about all precautions you need to take into account!

4.1. Gel preparation

1. Clean gel tray, comb and buffer tank with ethanol. Rinse thoroughly with deionized water. Rinse again with ethanol and let air dry.
2. Clamp the gel tray in its holder and insert comb.

3. For a 1.5% 60 mL gel, add 60 mL 1x TBE buffer to 0.9 g agarose.
4. Melt in the microwave (approx. 1 minute at power stand “7”).
5. After cooling down to approx. 60°C, add 6 µL GelRed. *GelRed should be added in the red delineated zone under the fume hood in lab 8 while wearing PURPLE nitril gloves and using the Eppendorf Reference pipette that is located in the red delineated zone in lab 8.*
6. Swirl gently to mix the GelRed into the gel solution.
7. Pour the gel in the tray.
8. Let gel solidify during approx. 15-20 minutes.

4.2 Sample preparation

9. Take a sample containing approx. 0.2 to 0.5 µg DNA (typically about 1-3 µL)
10. Add nuclease free-water to a final volume of 5 µL and add 1 µL loading dye. Do this preferably in a 0.2 mL PCR tube. Mix by pippeting up and down.
11. Briefly spin the samples them to collect them at the bottom of the tube.

4.3 Gel loading and running

12. Unclamp gel tray from its holder and place it in the buffer tank;
13. Remove comb.
14. Fill buffer tank with 1x TBE buffer such that the gel is just covered in it. Make sure the buffer fills the slots made by the comb.
15. Load the samples on the gel.
16. Include a ladder on your gel. The ladder is ready-to-use and 6 µL can be pipetted directly into a slot.
17. Close the lid of the buffer tank.
18. Attach red and black power cords to the power source.
19. Run the gel at 50V for 10 minutes. Afterwards switch to 100V for another 20 minutes (or until the bromophenol blue in the Loading Buffer migrated through about 2/3 of the gel). Since DNA has a negative charge it will migrate through the gel from the negative (black) to the positive (red) pole.
20. Turn off the power source when finished.

4.4. Gel scanning

Note: Again, it is strongly suggested to contact someone who did this before as certain precautions need to be taken into account for your own safety and the safety of others!

1. Wear PURPLE nitril gloves.
2. Turn on the Gel Doc and open the cabinet.
3. Take the gel tray out of the buffer tank, blot it dry with a paper towel and walk it over to the gel Doc.
4. Carefully let the gel slide of the tray onto the cabinet.
5. Close the cabinet and turn on the trans-UV light by pushing the button.
6. Take off your gloves!!!
7. Turn on the computer and start the Quantity One Software by clicking on the short-cut on the desktop.
8. Go to file > Gel Doc
9. Check “UV” in “image mode”.
10. Click on “auto-exposure”. An image of your gel will steadily appear. If the software is about to expose the gel to long, interrupt the auto-expose by clicking the “freeze” button. An alternate method is by choosing for “manual exposure” and adapt the exposure time manually.
11. You can optimize and annotate your image if you like.
12. Save your image.
13. Turn off the computer.
14. Wear again PURPLE nitril gloves
15. Open the cabinet and remove the gel. Dispose it in the ‘risicohoudend medisch afval’ waste container (yellow bin under the Gel-Doc).
16. Take off one glove
17. Clean the cabinet tray with deionized water *(ungloved hand)* and some paper towel *(gloved hand)*. Dispose this towel in the ‘risicohoudend medisch afval’ waste container (yellow bin under the Gel Doc).
18. Close the cabinet and turn off the Gel Doc.

19. Dispose your glove in the ‘risicohoudend medisch afval’ waste container (yellow bin under the Gel Doc).

4.5 Gel interpretation

Intact total DNA will show one sharp band above the first band of the ladder loaded on your gel (Figure 1A). If other bands are observed on the lower part of your gel, this might indicate RNA contamination (Figure 1B). A smear indicated degraded DNA.

PCR amplified DNA should show a clear band with expected size (size of the amplicon). The size can be relatively deduced from the markings of the loaded ladder.

4.6. Clean up

Clean, gel tray, comb and buffer tank with soap. You can use the bucket under the fume hood for this. Rinse with deionized water and dry with paper towel. *Make sure not to touch the squirt bottle with deionized water with contaminated gloves!!*

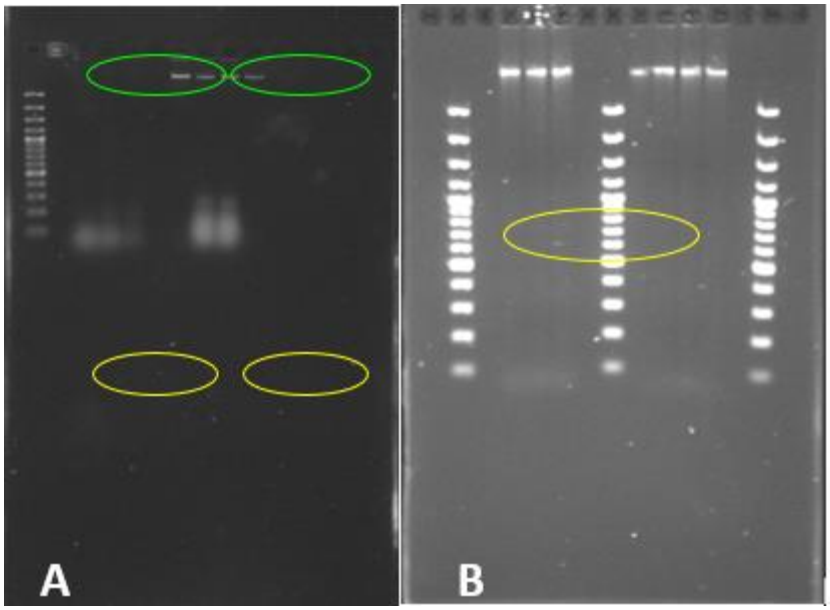


Figure 1 A: Good DNA extracts with genomic DNA (green circles) and little or no RNA present (yellow circles). **B:** Excessive RNA present (in the yellow circle) in the DNA extract

Appendix 6. NanoDrop measurements of the extracted eDNA samples.

Station	Date of Sampling	eDNA concentration (ng/uL)	A260/A280 ratio	A260/A230 ratio
120	14-Oct-14	NS	NS	NS
215	14-Oct-14	0.300	-0.580	-0.130
ZG02	14-Oct-14	NS	NS	NS
130	14-Oct-14	-10.600	1.280	0.310
230	14-Oct-14	5.000	2.040	-0.190
330	14-Oct-14	13.500	1.940	2.040
700	14-Oct-14	3.100	2.830	1.560
710	14-Oct-14	2.700	1.940	-0.110
780	14-Oct-14	10.800	1.700	-0.540
120	24-Mar-15	57.900	1.930	-25.760
215	24-Mar-15	39.700	2.090	2.370
ZG02	24-Mar-15	27.400	2.050	3.160
130	24-Mar-15	13.900	1.830	-0.830
230	24-Mar-15	20.300	1.890	-1.240
330	24-Mar-15	43.000	1.990	2.820
700	24-Mar-15	15.700	1.640	-1.560
710	24-Mar-15	38.900	1.940	-4.490
780	24-Mar-15	25.000	1.890	-1.400
120	19-May-15	29.300	2.050	-1.750
215	19-May-15	NS	NS	NS
ZG02	19-May-15	45.900	1.980	4.320
130	19-May-15	34.600	1.500	24.440
230	19-May-15	4.500	1.580	-0.170
330	19-May-15	9.900	1.770	-0.460
700	19-May-15	6.300	1.600	-0.290
710	19-May-15	11.000	1.710	-0.550
780	19-May-15	14.300	2.040	-0.570
120	18-Aug-15	451.100	1.930	2.280
215	18-Aug-15	240.900	1.920	3.080
ZG02	18-Aug-15	147.400	1.890	4.180
130	18-Aug-15	499.500	1.880	2.210
230	18-Aug-15	179.500	1.880	3.920
330	18-Aug-15	47.200	1.930	-3.340
700	18-Aug-15	122.800	1.810	10.210
710	18-Aug-15	550.200	1.870	2.200

780	18-Aug-15	112.800	1.920	8.110
120	28-Oct-15	109.300	1.700	2.720
215	28-Oct-15	161.500	1.890	3.040
ZG02	28-Oct-15	57.700	1.900	22.730
130	NS	NS	NS	NS
230	28-Oct-15	405.000	1.480	0.810
330	28-Oct-15	30.800	1.900	-4.820
700	28-Oct-15	792.700	1.480	0.850
710	28-Oct-15	1375.000	1.670	1.080
780	28-Oct-15	33.300	1.830	3.570
120	28-Jan-16	26.200	1.770	-6.980
215	NS	NS	NS	NS
ZG02	28-Jan-16	9.500	1.350	-54.000
130	28-Jan-16	-435.800	1.420	3.560
230	28-Jan-16	-11.100	1.760	0.540
330	28-Jan-16	12.900	1.940	-0.590
700	28-Jan-16	630.000	1.220	1.000
710	28-Jan-16	71.200	1.560	1.230
780	28-Jan-16	356.700	1.210	1.190
120	30-Mar-16	182.800	1.830	2.180
215	30-Mar-16	44.000	1.940	3.380
ZG02	30-Mar-16	44.800	1.940	4.160
130	30-Mar-16	64.900	1.530	1.930
230	30-Mar-16	89.100	1.940	3.340
330	30-Mar-16	76.000	1.900	3.200
700	30-Mar-16	172.200	1.700	1.710
710	30-Mar-16	341.800	1.770	1.860
780	30-Mar-16	189.600	1.830	2.470
120	25-May-16	293.100	1.850	1.690
215	25-May-16	48.900	1.890	-19.110
ZG02	25-May-16	148.600	1.900	2.440
130	25-May-16	1464.400	1.580	1.180
230	25-May-16	2542.600	1.510	0.860
330	25-May-16	119.800	1.930	3.000
700	25-May-16	247.500	1.750	1.460
710	25-May-16	448.500	1.830	1.950
780	25-May-16	78.400	1.050	1.280
120	23-Aug-16	294.400	1.870	2.490
215	23-Aug-16	165.800	1.870	2.780

ZG02	23-Aug-16	-34.900	0.500	0.720
130	23-Aug-16	214.800	3.410	3.000
230	23-Aug-16	36.200	-1.150	-4.300
330	23-Aug-16	216.600	1.910	2.470
700	23-Aug-16	380.200	1.990	1.430
710	23-Aug-16	765.500	1.500	0.920
780	23-Aug-16	181.300	1.970	2.310

Appendix 7. *C. hysoscella* eDNA concentrations (in g/L and in pg/L) from each station across the sampling months.

Sampling time	Station	Replicates			Mean	STDEV	eDNA concentration (g/L)	eDNA concentration (pg/L)	STDEV pg/L
		1	2	3					
Oct-14	120	NS	NS	NS	NS	NS	NS	NS	NS
Oct-14	215	2.51E-12	2.51E-12	2.45E-11	9.85E-12	1.2708E-11	9.851E-15	0.009851	0.012708057
Oct-14	ZG02	NS	NS	NS	NS	NS	NS	NS	NS
Oct-14	130	4.59E-33	1.71E-26	7.97E-29	5.73E-27	9.8498E-27	5.72656E-30	5.72656E-18	9.84977E-18
Oct-14	230	2.49E-28	5.69E-22	2.40E-20	8.19E-21	1.3704E-20	8.19475E-24	8.19475E-12	1.37037E-11
Oct-14	330	3.35E-17	1.08E-15	1.41E-15	8.42E-16	7.1979E-16	8.42293E-19	8.42293E-07	7.19785E-07
Oct-14	700	8.95E-14	3.57E-13	5.67E-12	2.04E-12	3.1476E-12	2.03873E-15	0.00203873	0.003147609
Oct-14	710	-	-	-	-	-	-	-	-
Oct-14	780	4.05E-28	9.15E-28	1.68E-22	5.60E-23	9.6994E-23	5.60004E-26	5.60004E-14	9.69945E-14
Mar-15	120	1.01E-21	1.24E-23	2.63E-27	3.39E-22	5.772E-22	3.39E-25	3.39E-13	5.77198E-13
Mar-15	215	2.16E-19	4.87E-19	6.42E-17	2.16E-17	3.6854E-17	2.16E-20	2.16E-08	3.68545E-08
Mar-15	ZG02	5.88E-20	8.98E-24	4.72E-21	2.12E-20	3.2642E-20	2.12E-23	2.12E-11	3.26424E-11
Mar-15	130	-	2.25E-47	1.99E-55	1.13E-47	1.5942E-47	1.13E-50	1.13E-38	1.59417E-38
Mar-15	230	2.34E-42	5.19E-38	4.11E-45	1.73E-38	2.999E-38	1.73E-41	1.73E-29	2.99898E-29
Mar-15	330	7.04E-24	7.27E-22	2.46E-21	1.07E-21	1.2617E-21	1.07E-24	1.07E-12	1.26175E-12
Mar-15	700	-	5.52E-47	9.62E-53	2.76E-47	3.9022E-47	2.76E-50	2.76E-38	3.90216E-38
Mar-15	710	6.54E-44	2.28E-31	3.80E-32	8.86E-32	1.2206E-31	8.86E-35	8.86E-23	1.22059E-22
Mar-15	780	5.38E-23	1.10E-18	4.49E-19	5.16E-19	5.5247E-19	5.16E-22	5.16E-10	5.52471E-10
May-15	120	1.15E-22	-	-	1.15E-22	NA	1.15E-25	1.15E-13	NA
May-15	215	NS	NS	NS	NS	NS	NS	NS	NS
May-15	ZG02	4.05E-24	4.04E-23	1.21E-24	1.52E-23	2.1876E-23	1.52E-26	1.52331E-14	2.1876E-14
May-15	130	-	-	-	-	-	-	-	-
May-15	230	-	-	2.30E-19	2.30E-19	NA	2.30E-22	2.301E-10	NA
May-15	330	1.12E-20	-	-	1.12E-20	NA	1.12E-23	1.12095E-11	NA

May-15	700	-	-	-	-	-	-	-	-
May-15	710	1.32E-19	-	1.17E-18	6.51E-19	7.3461E-19	6.51E-22	6.5115E-10	7.34613E-10
May-15	780	-	1.74E-19	-	1.74E-19	NA	1.74E-22	1.74E-10	NA
Aug-15	120	-	-	-	-	-	-	-	-
Aug-15	215	-	-	-	-	-	-	-	-
Aug-15	ZG02	8.71E-23	3.50E-19	2.64E-18	9.97E-19	1.434E-18	9.97E-22	9.96529E-10	1.43397E-09
Aug-15	130	-	-	5.64E-13	5.64E-13	NA	5.64E-16	0.0005643	NA
Aug-15	230	8.33E-24	-	-	8.33E-24	NA	8.33E-27	8.325E-15	NA
Aug-15	330	1.88E-09	3.69E-09	3.52E-09	3.03E-09	9.9983E-10	3.03E-12	3.0315	0.999834111
Aug-15	700	2.37E-24	4.65E-23	3.81E-15	1.27E-15	2.2014E-15	1.27E-18	1.271E-06	2.20144E-06
Aug-15	710	-	-	-	-	-	-	-	-
Aug-15	780	1.12E-18	9.26E-18	7.17E-18	5.85E-18	4.2312E-18	5.85E-21	5.852E-09	4.23121E-09
Oct-15	120	-	-	-	-	-	-	-	-
Oct-15	215	-	2.16E-19	2.55E-18	1.38E-18	1.6522E-18	1.38E-21	1.38473E-09	1.65219E-09
Oct-15	ZG02	-	4.62E-20	-	4.62E-20	NA	4.62E-23	4.6155E-11	NA
Oct-15	130	NS	NS	NS	NS	NS	NS	NS	NS
Oct-15	230	-	-	-	-	-	-	-	-
Oct-15	330	-	5.68E-20	-	5.68E-20	NA	5.68E-23	5.6805E-11	NA
Oct-15	700	-	-	-	-	-	-	-	-
Oct-15	710	-	-	-	-	-	-	-	-
Oct-15	780	-	-	-	-	-	-	-	-
Jan-16	120	-	-	-	-	-	-	-	-
Jan-16	215	NS	NS	NS	NS	NS	NS	NS	NS
Jan-16	ZG02	7.64E-21	2.35E-22	-	3.94E-21	5.2349E-21	3.94E-24	3.93638E-12	5.23489E-12
Jan-16	130	-	-	-	-	-	-	-	-
Jan-16	230	1.28E-18	1.94E-18	3.54E-17	1.29E-17	1.9513E-17	1.28716E-20	1.28716E-08	1.95129E-08
Jan-16	330	1.97E-14	1.91E-15	9.18E-15	1.02E-14	8.9168E-15	1.02475E-17	1.02475E-05	8.9168E-06
Jan-16	700	-	-	-	-	-	-	-	-
Jan-16	710	-	-	-	-	-	-	-	-
Jan-16	780	-	-	-	-	-	-	-	-

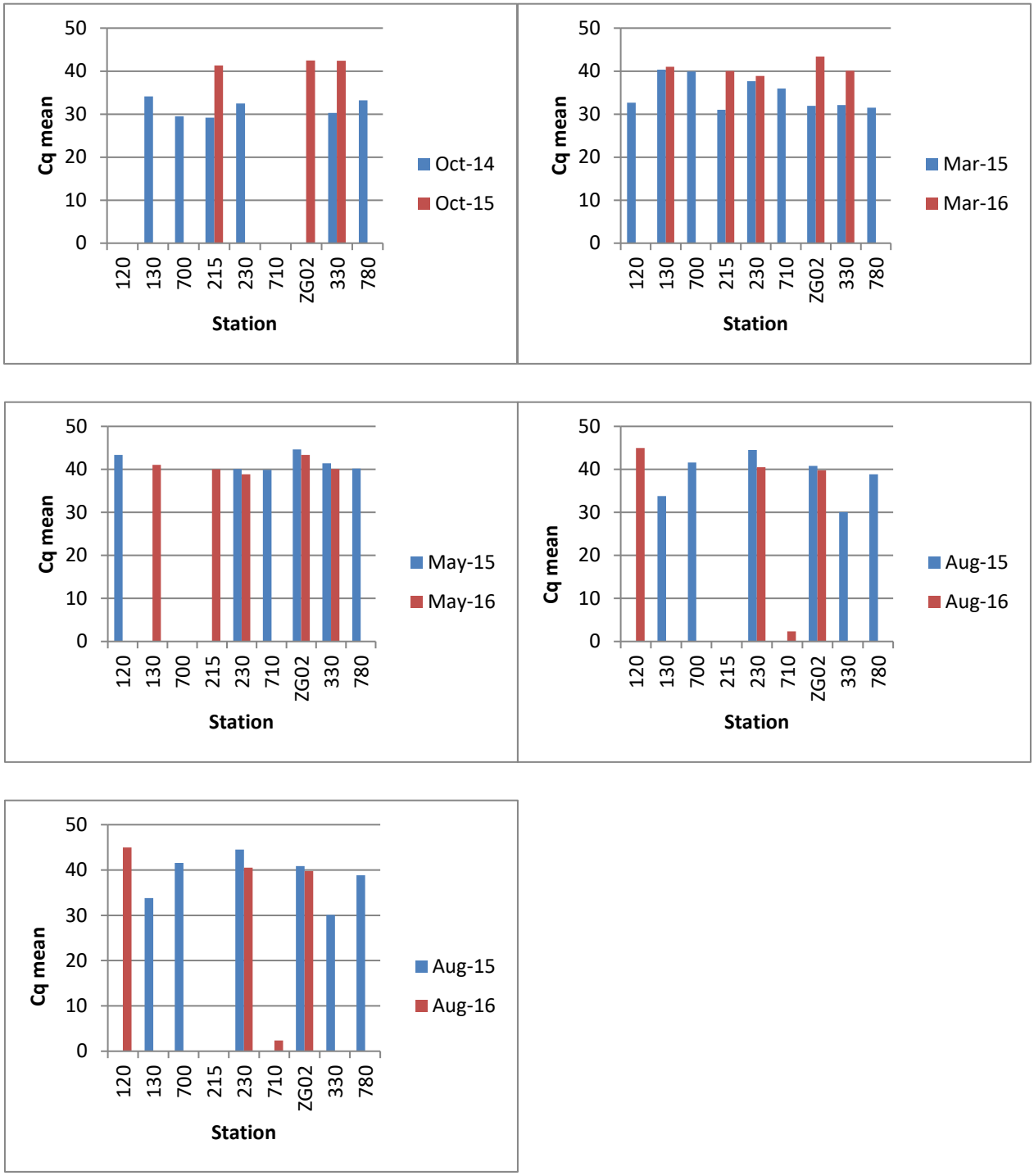
Mar-16	120	-	-	-	-	-	-	-	-
Mar-16	215	6.69E-14	8.73E-14	2.09E-14	5.84E-14	3.3982E-14	5.8355E-17	0.000058355	3.39823E-05
Mar-16	ZG02	-	-	8.26E-16	8.26E-16	NA	8.2575E-19	8.2575E-07	NA
Mar-16	130	1.45E-14	1.07E-14	1.88E-14	1.47E-14	4.0098E-15	1.46875E-17	1.46875E-05	4.00981E-06
Mar-16	230	7.55E-14	7.81E-13	1.37E-13	3.31E-13	3.9074E-13	3.3094E-16	0.00033094	0.000390743
Mar-16	330	2.58E-15	4.21E-13	8.42E-14	1.69E-13	2.2184E-13	1.69264E-16	0.000169264	0.000221836
Mar-16	700	-	-	-	-	-	-	-	-
Mar-16	710	-	-	-	-	-	-	-	-
Mar-16	780	-	-	-	-	-	-	-	-
May-16	120	-	-	-	-	-	-	-	-
May-16	215	9.88E-15	-	5.33E-15	7.60E-15	3.217E-15	7.60125E-18	7.60125E-06	3.21698E-06
May-16	ZG02	-	1.25E-15	-	1.25E-15	NA	1.2462E-18	1.2462E-06	NA
May-16	130	-	-	-	-	-	-	-	-
May-16	230	-	-	-	-	-	-	-	-
May-16	330	-	-	-	-	-	-	-	-
May-16	700	-	-	-	-	-	-	-	-
May-16	710	-	-	-	-	-	-	-	-
May-16	780	-	-	-	-	-	-	-	-
Aug-16	120	3.92E-19	-	-	3.92E-19	NA	3.9225E-22	3.9225E-10	NA
Aug-16	215	-	-	-	-	-	-	-	-
Aug-16	ZG02	1.15E-16	9.03E-18	1.13E-15	4.19E-16	6.2048E-16	4.18949E-19	4.18949E-07	6.20481E-07
Aug-16	130	-	-	-	-	-	-	-	-
Aug-16	230	1.96E-17	1.20E-16	-	6.98E-17	7.1064E-17	6.981E-20	6.981E-08	7.10642E-08
Aug-16	330	-	-	-	-	-	-	-	-
Aug-16	700	-	-	-	-	-	-	-	-
Aug-16	710	-	-	2.99E+01	2.99E+01	NA	0.029865	29865000000	NA
Aug-16	780	-	-	-	-	-	-	-	-

Appendix 8. Temperature profile of the different stations across the different sampling months.

Sampling time	Station and Temperature (°C)									Average temperature (°C) per month	Standard deviation of the temperature (°C) per month
	120	130	700	215	230	710	ZG02	330	780		
Oct-14	-	15.60	-	16.50	15.97	-	-	16.10	15.70	15.97	0.36
Mar-15	7.16	6.60	6.40	-	6.60	6.50	7.10	6.65	6.40	6.68	0.30
May-15	13.30	13.70	13.30	12.40	13.60	13.30	12.20	12.60	13.10	13.06	0.53
Aug-15	18.90	19.60	19.70	19.20	19.60	19.50	18.90	19.10	19.40	19.32	0.31
Oct-15	12.72	12.34	12.71	13.75	12.73	12.86	13.65	13.53	13.09	13.04	0.49
Jan-16	8.10	7.70	6.41	-	8.09	6.46	8.96	7.61	6.44	7.47	0.95
Mar-16	8.66	8.39	8.25	8.38	8.33	8.20	8.44	8.05	7.91	8.29	0.22
May-16	14.16	14.18	14.08	13.54	14.15	13.55	13.10	13.13	13.46	13.71	0.44
Aug-16	19.40	18.99	19.55	18.76	18.80	19.40	18.63	18.97	19.38	19.10	0.34
Average temperature (°C) per station	12.80	13.01	12.55	14.65	13.10	12.47	12.62	12.86	12.76		
Standard deviation of the temperature (°C) per station	4.69	4.73	5.31	3.82	4.67	5.20	4.45	4.70	5.01		

Note: - means no temperature was recorded

Appendix 9. Bar graphs comparing *C. hysoscella* eDNA detection and Cq mean for selected sampling months.



Note: No bar means no detection or no sample analyzed.