Masteroppgave

NTNU
Norges teknisk-naturvitenskapelige universitet
Fakultet for naturvitenskap
Institutt for biologi

Hedda Førde

Glow and Know – Evaluating the Potential of Spectral Bioluminescence as a Tool for Zooplankton Identification *in situ*

Masteroppgave i Ocean Resources
Veileder: Stephen Grant
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Sammendrag

Mange arter forsvinner i det stille, men dette er i ferd med å bli en av de største truslene vi står overfor i dag. For å begrense tapet av biologisk mangfold er det viktig å overvåke og forstå artene som lever i de ulike økosystemene. Dyreplankton er en divers gruppe organismer som finnes over hele kloden. De er bindeleddet mellom planteplankton, og de høyere konsumentene. Denne gruppen er derfor sentral for å forstå biologisk mangfold, populasjonsendringer og økosystemfunksjonalitet. Metodene som brukes for å overvåke dyreplankton i dag kan imidlertid være upresise og langsomme. Dermed er effektive metoder avgjørende for å bevare biodiversitet. Bioluminescens finnes i alle verdenshavene i ulike grupper dyreplankton. Tidligere forskning har vist at mye kan bli lært om økologien til en art ved å forstå deres bioluminescens. Det har også blitt antydet at bioluminescens kan ha taxa-spesifikke egenskaper, men det er usikkert til hvilken grad det kan brukes som et verktøy.

Målet med denne studien er å vurdere om dyreplanktonets emitterte lysspekter (spektrale bioluminescens), det vil si fargen på lyset, kan brukes til *in situ*-identifikasjon av taxa. Individer til målingene ble funnet med planktonhåv og diverse håndholdte håver, under polarnatt i Kongsfjorden, Svalbard, og ulike steder og årstider i Trondheimsfjorden, Midt-Norge. Variasjonen i spektral bioluminescens mellom, og innen, arter ble analysert, hvor taxa, måned, sted og stimulusmetode ble sammenlignet. Levende dyr ble stimulert, enten mekanisk, ved hjelp av lys eller osmotisk stress. Lyset ble umiddelbart målt med et spektrometer. Bølgetoppen (λ_{max}) og full båndbredde ved halv intensitet (FWHM) samt formen på lysspektrene ble sammenlignet. Individer ble morfologisk og molekylært artsbestemt til lavest mulig taksonomisk nivå.

Totalt ble det utført målinger på 270 individer, som tilhørte 21 ulike taksa, klassifisert til 10 arter, fem slekter, tre ordener, én klasse, én fylum, og noen eksemplarer i samlebetegnelsen Coelenterata (Cnidaria og Ctenophora). Bioluminescens ble innhentet fra 18 taxa tilhørende phyla Ctenophora, Cnidaria, Arthropoda og Annelida. Den hyppigste og vellykkede metoden for å stimulere dyrene, var med mekanisk stimulering. Noen få av artene hadde et unik spektra sammenlignet med de andre artene, og kunne skilles fra resten, som kopepoden, *Aetideopsis armata*, ulike euphausiider og *Clytia* spp. De bioluminescerende artene med λ_{max} i det blå fargespektret, ctenophorer, *Metridia* spp. og *Tomopteris* spp. var svært like og vanskelige å skille fra hverandre. Både *Metridia* spp. og *Tomopteris* spp. hadde imidlertid en annen spektralform enn ctenophorer. Den eneste intraspesifikke variasjonen ble funnet hos hydrozoa *Clytia* spp. som ble samlet i august i Trondheimsfjorden og i oktober i Hopavågen. Dette kan skyldes at de grønne fluorescerende proteinene uttrykkes ulikt i de to habitatene, eller at det er ulike arter de ulike stedene. Selv om det er variasjon mellom enkelte taxa, er det tvilsomt om det er praktisk mulig å få til *in situ*-målinger. Det er to hovedgrunner til dette. Lyssignalet er ofte svakt og skjer fort, noe som gjør det vanskelig å måle nært nokk. For det andre, har spektralmålingene fra studier vært svært varierende. Det er usikkert til hvilken grad dette skyldes biologiske variasjoner, som genetisk variasjon eller fenotypisk plastisitet, i taxaene, eller inkonsekvente metoder mellom studiene, f.eks. feilklassifiserte arter eller spektrometer med lav oppløsning. Dette understreker behovet for en taxa-spesifikk protokoll samt ytterlig kartlegging av spektral bioluminescens, på artsnivå. Når disse utfordringene er løst, kan bioluminescens bidra til å bevare det biologiske mangfoldet.

Abstract

Many species are quietly lost, which is becoming one of the biggest threats we are facing today. To restrain biodiversity loss, monitoring and understanding the species that live in various ecosystems has become an important task to conquer. Zooplankton is a diverse group of organisms found across the globe, connecting the phytoplankton and the higher consumers. This important group is thus central in understanding diversity, population changes and functionality of marine ecosystems. However, the methods used today to monitor zooplankton diversity can be unprecise and slow, hence, advanced techniques are needed. Bioluminescence is a ubiquitous trait of the world's oceans and is found in several zooplankton taxa. Previous research has shown that by understanding the bioluminescent flash of a species, much can be learnt about their ecology. It has also been shown that bioluminescence has taxa specific traits, but it is uncertain to what extent this can be used as a taxa recognition tool. The aim of this study is to assess if zooplankton spectral bioluminescence, meaning the color of the flashes, can be used for *in situ* taxa identification. Specimens for the measurements were collected with plankton nets and custom-made handheld nets from Kongsfjord, Svalbard, during polar night, and various locations of the Trondheimsfjord, mid Norway, during different seasons. The inter- and intraspecific variation in bioluminescent spectra was considered, where taxa, month, location and method of stimuli were compared. Bioluminescence was stimulated in live animals either mechanically, by light or by osmotic stress. The flashes were immediately recorded with a spectrometer. The peak wavelength (λ_{max}) and full bandwidth at half intensity (FWHM) as well as the general shape of the bioluminescence spectra were compared within and between taxa. Morphological and molecular identification was used to identify the specimens.

In total, measurements were performed on 270 specimens, belonging to 21 different taxa classified to 10 species, five genus, three orders, one class, one phylum, and some specimens to the collective term, Coelenterata (phyla Cnidaria and Ctenophora). Bioluminescence spectra were obtained from 18 taxa from the phyla Ctenophora, Cnidaria, Arthropoda and Annelida. The most successful method to trigger bioluminescence was mechanical stimulation. A few of the taxa had unique spectral composition compared to the other species, and were distinguishable from the rest, like the copepod *Aetideopsis armata*, various euphausiids and the hydrozoan *Clytia* spp. The bioluminescent specimens with a λ_{max} in the blue wavelength range, the ctenophores, *Metridia* spp. and *Tomopteris* spp., had very similar spectra, difficult to distinguish from each other. However, both *Metridia* spp. and *Tomopteris* spp. had a different spectral shape compared to the ctenophores. The only intraspecific variation was found in the hydrozoan *Clytia* spp., collected during August in Trondheimsfjord, and October in Hopavågen. This may be due to its green fluorescent proteins being expressed differently in the two habitats, or that it is variation due to two different species. Although there is variation between some taxa, the practicality of obtaining *in situ* measurements is questionable. This is for two reasons. Firstly, that the flashes are often dim and sudden, posing a proximity challenge. Secondly, the spectral measurements from other studies were highly variable. It is uncertain to what extent this is due to biological variations, like genetic variation or phenotypic plasticity, in the taxa, or inconsistencies in the spectral measurement methods, i.e. wrongly classified species or measurement devices with a low resolution. This stresses the need for a taxa specific protocol as well as further mapping spectral bioluminescence in more specimens, to species level. Once these challenges are solved, bioluminescence can ultimately help conserve biodiversity.

Acknowledgements

This thesis is a part of a project called *LightLife- Light as a Que for Life in the Arctic,* funded by The Norwegian Research Council. It was written at Trondheim Biological Station (TBS), in the Institute of Biology at the Norwegian University of Science and Technology (NTNU). My main supervisor is researcher Dr. Stephen Grant, and the co-supervisors are researcher Dr. Sanna Majaneva, researcher Dr. Martta Viljanen and professor Geir Johnsen.

As a starter, I want to thank my supervisors, Stephen, Sanna and Martta for everything. I feel that you have been present when I needed it, listened to me, and helped me in a patient way, despite how busy you are. This has created a very safe and comfortable learning environment that has allowed me to challenge myself, as well as enjoy (most of) the different steps during the last 1,5 years. Thank you so much Stephen, for spending time on creating the MATLAB codes, this has been far beyond my competence.

Secondly, thank you to everyone who has shown great interest or helped me with my work. Geir, thank you for letting me use your QEPro, join your fieldwork, and importantly, motivating me with your contagious curiosity for this thesis. Mari Ann Østensen, thank you for teaching me your ways in the lab, and assisting me with the last taxa that needed to be identified when my time was running short. Ragnhild Irene Jacobsen and Nadia van Eekelen, you helped me with some of my fieldwork and measurements, and importantly, understanding the world of the «difficult» bioluminescent genus, *Tomopteris*. It was always a blast working with you, and I really appreciated all the laughs. Taran Five, you were my co-master student in the LightLife project, and it has been a pleasure working with you. It has truly eased my way into the thesis life, and it was particularly fun when we went on adventures in Svalbard, and in Oslo for the Svalbard Science Conference.

Dear family, you were always a phone call away when things got hard, or I needed to share a happy moment. Eivind, your presence and all the support has meant a tremendous amount. TBS girls, we became a good and supportive group, and I truly hope we will stay in touch. I won't forget our breakdown tally-count and the support we gave to each other. Dear Els, if it wasn't for all the dips in sea, the runs up the hill, your cat memes, and the friendship we developed, this process would not have been the same.

Lastly, I want to add that I really enjoyed working on this project, and I have learned so much. I put my soul into this, and I hope the research one day will come of use to someone out there.

Best wishes,

Trondheim, May 2024

Hedda Førde

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List of Abbreviations and Symbols

ALAN	Artificial light at night
ANOVA	Analysis of Variance
BLAST	Basic Local Alignment Search Tool
CBD	Convention on Biological Diversity
cf.	Confer, “compare with”
DVM	Diel vertical migration
eDNA	Environmental DNA
EtOH	Ethanol
FAO	Food and Agriculture Organisation of the United Nations
FWHM	Full width half maximum
GBIF	Global biodiversity Facility
GFP	Green fluorescent protein
H0	Null hypothesis
ID	Identification
IMR	Institute of Marine Research
IPBES	Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services
KCl	Potassium chloride
n	Number (often number of specimens)
NA	Not available
NAC	North Atlantic Current
NTNU	Norwegian University of Science and Technology
OMA	Optical multichannel analyser
OSPAR	Oslo-Paris Convention
PCR	Polymerase chain reaction
PIRNO	Knipovich Polar Research Institute of Marine Fisheries and Oceanography
QQ plot	Quantile-quantile plot
SBL	Spectral bioluminescence specimen ID
SE	Standard error
SEM	Standard error of mean
SNR	Signal to noise ratio
UiO	University of Oslo
UNEP	United Nations Environmental Program
UNFCCC	United Nations
UV	Ultraviolet
WP	Work package
λ_{max}	Wavelength at peak intensity

1 Introduction

1.1 The biodiversity crisis

Biodiversity loss is one of the three central challenges our environment is facing today. This threat, together with climate change and pollution are collectively known as the triple planetary crisis (UNFCCC, 2022). Biodiversity is short for biological diversity and is defined as the variability and variety of biological organisms in all its forms, from genes to species, populations and entire ecosystems (Wilson & Peter, 1988). This includes all varieties of life that has evolved over 4,5 billion years, with some occurring in the past and some today. To preserve biodiversity, the roots of the challenges need to be found on both a global and local scale. The health of the whole ecosystem, organisms and how they interact with their habitat, depends on how we tackle the triple planetary crisis.

According to the United Nations Environmental program (UNEP), there are five main drivers that threaten biodiversity and ecosystems today (UNEP, 2022). (1) Invasive species; this has contributed to 40% of species extinction since the 17th century (CBD, 2006). (2) Changes in land and sea use; which is often regarded as the biggest threat for biodiversity, where the impact from the global food system are the most pressing (Benton *et al.*, 2021). (3) Climate change; where changing temperatures are already affecting some of the most vulnerable habitats, for example the sea ice habitats in the Arctic (CBD, 2018). (4) Pollution; which is particularly pressing the marine and freshwater habitats (IPBES Secreteriat). (5) Direct exploitation of natural resources; for example, overfishing or trade in ornamental species (UNEP, 2022). However, according to a report by UNEP and Food and Agriculture Organisation of the United Nations (FAO), there is hope for today's society to become "#GenerationRestoration", meaning the ones who manage to make the necessary changes for a sustainable future (UNEP & FAO, 2021). The report discusses priority areas, the places where conservation can have the largest benefits for biodiversity and climate stabilization. If only 15% of ecosystems in priority areas are restored, it could halt 60% of expected species extinction, preserving biodiversity.

Humanity is already noticing the repercussions of overexploited ecosystems (UNEP & FAO, 2021). Functioning ecosystems are central for humans due to their ecosystem services; the benefits people obtain from ecosystems (NatureScot, 2023). For example, the oceans biological carbon pump is one of Earth's most valuable ecosystem services because it helps regulate the atmospheric climate and its ability to take up CO_2 (Jin *et al.*, 2020). Therefore, because of ecosystems and biodiversitys' important functions for humans, preserving natural resources is important role nations need to venture. This can be done through global policies, like The Kunming-Montreal Global Biodiversity Framework (GFB) from 2022, which was established to protect life on Earth. This historical agreement fronts a framework consisting of a biodiversity plan with global targets and goals to be reached by 2030 and 2050. To achieve these goals, primary research is needed to fully understand biodiversity in local regions.

1.2 Light and life in the dark seas

1.2.1 Light pollution can threaten biodiversity

Over millennia, there has been natural changes in Earth's climatic variables, like CO_2, temperature, and ice coverage. Light from the sun has been a rhythmic and harmonic occurrence, changing with the moon and Earth's alignment in the solar system (Cohen *et al*., 2020). This has defined the light climate of our ecosystems and organisms, referring to the intensity, spectrum, and duration of light at a given location. For example, in the northern hemisphere, above the Arctic circle, the sun does not cross the horizon during winter, characterizing the polar night with no sunlight over several months (Berge *et al*., 2015). On the other hand, during summer, the sun does not set at all, and organisms living in these conditions have adapted to these extreme, yet predictable, light changes. Today, light pollution is changing these natural light climates and is becoming an emerging concern for biodiversity.

Light pollution, or specifically ecological light pollution, is the artificial light that changes the natural light and dark patterns in an ecosystem (Longcore & Rich, 2004). There is on average a 6% yearly increase in artificial light at night (ALAN), the period when its impact is most destructive for organisms. This makes it one of the fastest spreading environmental challenges of the Anthropocene (Hölker *et al*., 2010a, Hölker *et al*., 2010b). Despite of limited studies focusing on the impact of artificial light on marine ecosystems, it is known that light pollution can lead to long-term and short-term consequences for organisms, where both physiological and ecological consequences have been observed. One important physiological adaptation that relies on a stable light climate, is organisms' biological clocks, also known as circadian rhythm. Circadian rhythms are commonly dependent on day-night cycles, moon phases or seasons (Last *et al*., 2020). However, with increasing artificial light, these cycles can be affected, reducing the fitness of both animals and plants (Last *et al*., 2020). Nguyen *et al*. (2020) performed a laboratory study on how tropical copepods are affected by increasing ocean temperatures and increasing artificial light. They concluded that zooplankton fitness significantly decreased under both temperature and light stressors. These examples demonstrate how light pollution is of emerging concern and research focus is needed on the effects of a changing light climate.

Ecological light pollution is also increasing with thinning sea ice and coastal darkening. As the sea ice decrease due to increasing sea surface and atmospheric temperature, more light penetrates the sea (Nicolaus *et al*., 2012). This is known to increase primary production (Frey *et al*., 2022), but it is uncertain how it affects other species in higher trophic levels, like zooplankton (Flores *et al*., 2023). Yet, it is predicted that zooplankton will migrate deeper, which can have consequences for the carbon fluxes and Arctic food web (Flores *et al*., 2023). Furthermore, coastal darkening, a process where there is a long-term reduction in water clarity, affects the optical light climate, often due to an increase in particulate matter (Aksnes *et al*., 2009). This is becoming an increasing problem in Norwegian coastal waters (Franze *et al*., 2023).

1.2.2 The light climate in ocean

Few dark corners in the world still exist, for example deep in the oceans. In the marine environment, the light climate drastically changes with depth (NOAA, 2023). The euphotic zone is where sunlight is still apparent, and photosynthesis can still occur. With optimal light conditions and few suspended particles in the ocean, the euphotic zone can be down to 200 m. In the twilight zone, beyond 200 m

depth and down to 1000 m, only a slight amount of light is evident, and photosynthesis no longer happens. No rays of the sun can reach greater depths below 1000 m (NOAA, 2023). Not only does the light intensity change by depth, but also light spectra, as reviewed by Webb (2023). The light spectrum changes by long wavelengths being absorbed at the shallower depths, and the shorter wavelengths travelling the furthest (Figure 1). The long wavelengths are red and yellow colors, while the short wavelengths are blue and violet colors. Figure *1* illustrates the differences in light penetration between coastal waters and the open ocean. It shows that light travels further in the open ocean, specifically blue light, compared to coastal waters, where green travels the furthest, but to a much shallower depth. The vast differences between the two waterbodies are because open oceans generally have fewer suspended particles and microorganisms that absorb light, in contrast to coastal waters. Phytoplankton and other photosynthetic organisms absorb the blue and red-light during photosynthesis. How far light travels and what wavelengths that are absorbed can greatly vary based on location and time of year. For example, Aksnes (2015) calculated the euphotic zone to be down to 15 m in Norwegian coastal waters, while 112 m in North Atlantic Ocean waters. This 98 m difference was due to less phytoplankton in the North Atlantic (Aksnes, 2015).

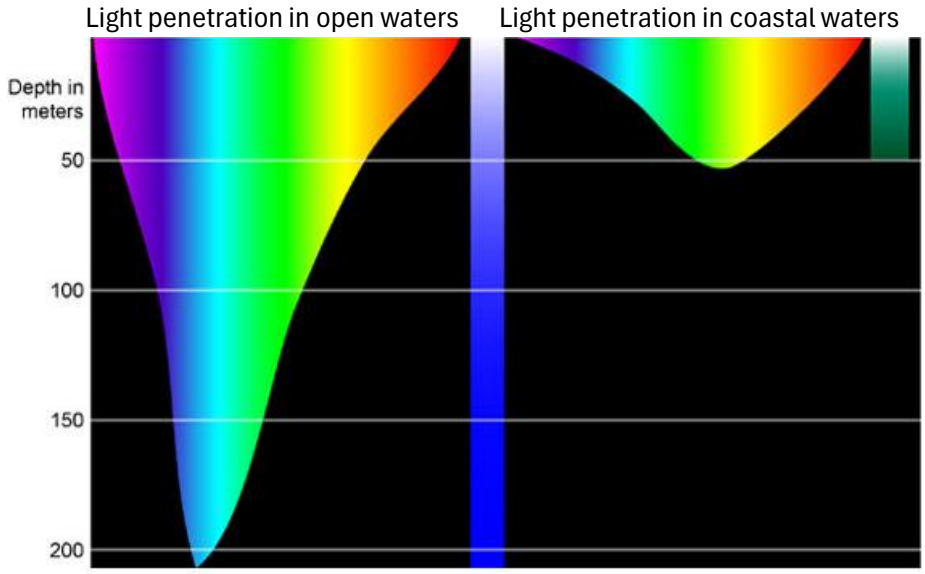

Figure 1: Light penetration in the open ocean and coastal waters. Blue light travels the furthest in the open ocean, down to 200 m depth. Red, orange, and yellow wavelengths are absorbed first. In coastal waters, green travels the furthest, down to 50 m. Short violet wavelengths are absorbed first. Note that this is a simplified example of light penetration, and the exact colors vary by suspended particles and microorganisms. Illustration by Kyle Carothers, National Oceanic and Atmospheric Administration (NOAA).

1.2.3 Organisms are adapted to the dark

How light travels in the ocean, is important to understand because it affects the light perceived by organisms at certain depths. Often, the vision of various species, also known as spectral sensitivity, is similar to the dominant light spectra that reaches the depth where the organism thrives. For example, the spectral peak in the Kongsfjord, Svalbard, during the polar night is 485 nm, while the spectral sensitivity of the Arctic krill (*Thysanoessa inermis)* living there is 492 nm (Cohen *et al*., 2015). This adaptation allows organisms to optimally see their environment, and is particularly important for vertical migration, predation and avoiding predators (Warrant & Locket, 2004). Furthermore, the

correlation between spectral peak in the ocean and spectral sensitivity in animals shows the finetuning between species and their environment.

The Arctic krill that thrives during the polar night, lives in a habitat that is comparable to the light climate of the deep sea (Berge *et al*., 2012). There are different types of polar night, ranging from the extreme winter months in the pole where there is no visible change in light, to clear dawn and dusk patterns (Berge *et al*., 2015). The Arctic Ocean is unique in this way because other oceans of the world experience less drastic changes, and the Southern Ocean by Antarctica, lies almost fully above the Antarctic circle. Previously, it was thought that biological activity in oceans during the polar night were at a resting state, but with increasing interest in Arctic research, the contrary has been shown (Berge *et al*., 2020a; Berge *et a*l., 2020b 2020; Berge *et al*., 2015). Newer studies have found a high abundance of interactions between species, and for some regions, juvenile activity is even higher during the winter months than in the sunlit months (Berge *et al*., 2020b). However, as a byproduct of the interest in the Arctic, light pollution in this pristine environment is an emerging concern, and research has shown that zooplankton can be affected down to 200 m depth (Berge *et al*., 2020a). The long-term effects, however, are still unknown.

Although there is no atmospheric light available in the deep sea or polar night, nor during "regular" nights across the world, there is still one important characteristic that can illuminate the sea: bioluminescence. This biological light is known to be the only source of visual light when certain depths are reached (Cohen *et al*., 2020).

1.3 Bioluminescence in marine ecosystems

Bioluminescence, or the production and emission of light by an organism, is often described as a ubiquitous trait; it is found all over the world's ocean, even though most species do not possess it(Claes *et al*., 2024; Widder, 2010). A comprehensive and new review found 31% of species from bioluminescent genera, were potentially bioluminescent – meaning light producing structures were found, or bioluminescence was seen (Claes *et al*., 2024). This covers around 1000 bioluminescent genera across the world. In addition, a study on marine biodiversity outside California found that 69% of the observed species were also most likely bioluminescent (Martini & Haddock, 2017). Bioluminescence has evolved independently over 40 times indicating that it is of high importance in multiple taxa and with a potential for much variation (Claes *et al*., 2024; Haddock *et al*., 2010; Letendre *et al*., 2024). Thanks to its fascinating displays, bioluminescence has sparked an interest since before the ancient philosophers BCE and up to the present (Letendre *et al*., 2024).

Fluorescence is another natural light phenomenon, but the molecules require photons to emit energy in the form of light. Green fluorescent proteins (GFPs) are an example of fluorescence, where photons can induce fluorescence. GFP is found for example in some cnidarians. Normally they have a bioluminescent signal with maximum around 470nm, but for the species with GFP, the bioluminescent light can sometimes induce fluorescence resulting in the light to be emitted as longer, greener, wavelengths (Haddock *et al*., 2010).

1.3.1 The purpose and mechanisms of bioluminescence

The likely reasons for why organisms are bioluminescent can be grouped into three categories: defense, offence, and intraspecific communication. For defense, the copepod *Metridia lucens* tends to aggregate and create bioluminescent hotspots, which is considered a minefield against predators (Widder, 2002). It has been hypothesized that when the predators pass through the bioluminescent minefield, their shadows will expose the predator to larger predators in the area and thus indirectly protect *M. lucens*. For both offense and defense, some species can counterilluminate, a property where organisms adjust their bioluminescence to match their background (Haddock *et al*., 2010). As Haddock *et al*. (2010) summarized, this is for example seen in some krill species (Order Euphasiiacea), where they adjust their ventral photophores through nervous control. Interestingly, some sharks (Order Squaliformes) have bioluminescent organs and can also counterilluminate. However, it may be that bioluminescence in Squaliformes serves a camouflaging purpose for offense instead of defense (Mallefet *et al*., 2021). The third reason, intraspecific communication, is well understood in terrestrial environments, like in bioluminescent fireflies (Family Lampyridae), but more difficult to study and understand in the ocean (Widder, 2010). It has been observed that schools of ponyfish (*Leiognathus splendens)* synchronize their bioluminescence to create extensive displays, lighting up the underwater visibility even for the human eye (Woodland *et al*., 2002). Although the mechanisms and reasons for this event are still unknown, it is likely that it can have a role in spatial organization, which can be interpreted as intraspecific communication, in relation to the location of planktonic prey or reduced predation.

The chemical reaction behind bioluminescence normally occurs in light producing cells, and some taxa have dedicated light organs called photophores (Haddock *et al*., 2010). In other species, the chemicals can be emitted as luminescent secretions where bioluminescence is seen outside of the organisms' body (Haddock *et al*., 2010). The general chemical pathway involves oxygen reacting with a taxa specific light sensitive molecule, catalyzed by enzymes (Wilson & Hastings, 2013). The light sensitive molecule is called luciferin and there are four known luciferin types in the ocean: bacterial, dinoflagellate, cypridina and coelenterazine (Haddock *et al*., 2010). The catalyzing enzymes are either luciferase or photoprotein. Furthermore, some species are dependent on obtaining luciferin or enzyme from their diet. This is most likely the case for euphausiids because they have the same luciferin as dinoflagellates (Nakamura *et al*., 1989). Some organisms have symbiotic bacteria that produce bioluminescence, for example in the "lightbulb" organ of the angler fish (Order Lophiiformes) and on the body of the ponyfish *L. splendens* (Haddock *et al*., 2010; Letendre *et al*., 2024). The luminescence that occurs due to symbionts, or when both the luciferin and luciferase are obtained from diet, is known as extrinsic luminescence. When it is controlled by an organisms' own chemicals, so independent of diet, it is intrinsic luminescence.

Several species can however have a varying bioluminescent signal. This can for instance change with optical filtering in cells, that different parts of the specimens body emits different colors, or due to variations throughout life stage (Haddock & Case, 1999). Both changes by optical filtering and life stage have been observed in the Australian pinecone fish, *Cleidopus gloria-maris*. During the juvenile life stages, the bioluminescent signal from the posterior to anterior photophoes range from a λ_{max} of 555 nm to 516 nm, respectively. However, in the adults the λ_{max} is at shorter wavelengths, with less

variation, ranging from 506 nm to 503 nm across the body (Widder *et al*., 1983). Furthermore, the light producing organ contains the bacteria *Vibrio fischeri*. When isolated, the bacteria have a λ_{max} of 492 nm (Fitzgerald, 1977). It is assumed that reddish-orange optical filtering is the reason for the change from green to blue-green when the bacteria are measured in intact photophores, versus when isolated (Haneda, 2015).

The bioluminescent color, also known as the spectral composition, is defined by a specific type of luciferin-luciferase complex, their associated proteins and other optical filtering in cells or tissue (Wilson & Hastings, 2013). The spectral composition of a bioluminescent signal affects how far the light travels and if other organisms can perceive it (Haddock & Case, 1999). As discussed above, blue travels the furthest in the ocean, and most taxa have a blue bioluminescent signal, as well as a peak spectral sensitivity around the blue wavelengths. A study by Frank and Widder (1999) led to the conclusion that vision in some crustaceans may be more closely adapted to bioluminescence than downwelling light, further stressing the importance of bioluminescence in marine habitats. Haddock and Case (1999) found that even though most signals are blue, the deep dwelling species tend to have shorter wavelengths compared to the shallower species. However, they also argued that spectral details may have small ecological significance because several exemptions have been found, like the yellow pelagic worm, *Tomopteris helgolandica* (Gouveneaux & Mallefet, 2013). Another aspect of a bioluminescent signal which can differ between taxa is the mechanisms of the flash. The copepod *M. lucens* for instance, has a flash characterized by a rapid increase in intensity and a slower decay lasting up to one minute (David & Conover, 1961). The ctenophore *Bolinopsis infundibulum* however, has a pulsating flash, increasing and decreasing in intensity (Krohn-Pettersen, 2023).

1.3.2 Measuring bioluminescence

Bioluminescent traits, like spectral composition and flash kinetics are commonly studied in relation to bioluminescence research. A bioluminescent spectrum can be measured and described by the parameters λ_{max} and full-width-half-maximum (FWHM). The λ_{max} is the wavelength (nm) at maximum intensity (measured in photons), while the FWHM is the full band width at half of the maximum intensity (nm). Flash kinetics is measured by the number of photons emitted over time during one flash. Studies have shown that there are differences in the flash kinetics of different species, making it a possible tool for taxa recognition based on bioluminescence (Johnsen *et al*., 2014; Krohn-Pettersen, 2023). Whereas Johnsen *et al*. (2014) suggested using flash kinetics as a potential tool for identification at species level, Krohn-Pettersen (2023) stated that identification was only possible at higher taxonomic levels.

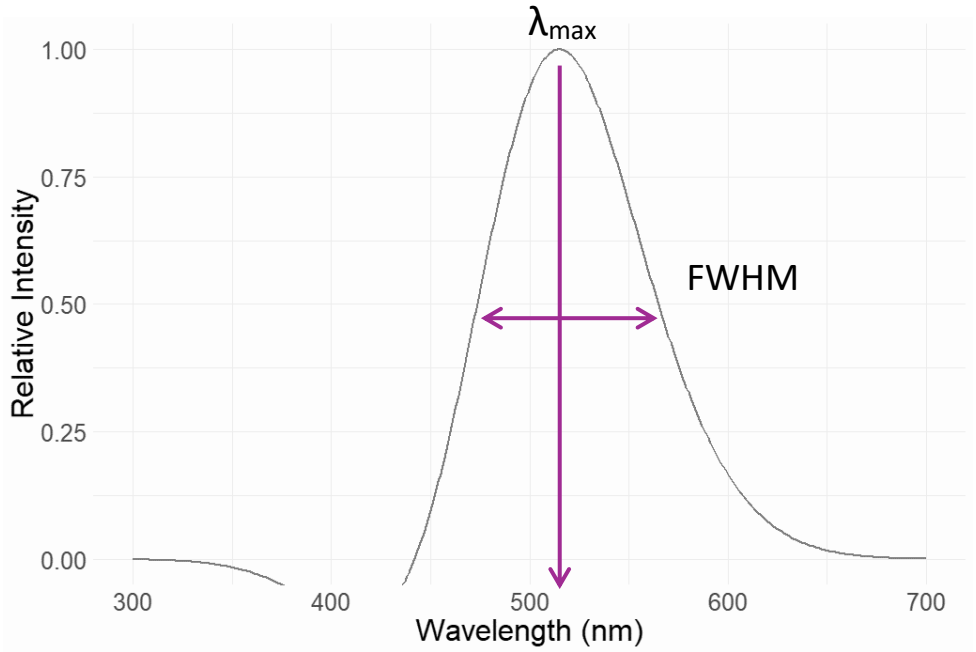

Figure 1: Illustration of a fictive bioluminescent spectra, with a peak wavelength (λ_{max}) of 515 nm, and full bandwidth at half intensity (FWHM) of 100 nm. Illustration created by random numbers in R studio, by Hedda Førde.

To be able to measure bioluminescence, the specimens first need to be stimulated to produce a flash. The maximum amount of light a specimen can emit is often referred to as bioluminescent potential (Letendre *et al*., 2024). The term is used inconsistently in literature (Letendre *et al*., 2024), thus in this context, it will be used to describe the ability and amount of light produced by one or more specimens. For maximum bioluminescent potential specimens need to be in an optimal state, meaning not damaged or recently stimulated (Letendre *et al*., 2024). During Haddock and Cases' (1999) experiment on luminescent wavelengths in gelatinous zooplankton, or specifically specimens in the phyla Cnidaria and Ctenophora (also known as coelenterates), they noted that a species can both reversibly and irreversibly lose their bioluminescence after exposure to light. One solution to this was dark adaptation, which normally allowed full recovery. Furthermore, taxa with the dinoflagellate luciferin are most likely affected by day-night cycles because the luciferin molecule originated from conditions where it would photosynthesize during the day and produce light during night (Haddock *et al*., 2010). In addition, some species are for example, only luminescent during certain seasons, or their bioluminescent potential is reduced due to dietary deficiency. Therefore, bioluminescent potential is central when studying the signals, as it can give an indication of the health of the specimens, but can also lead to "false negatives", where a bioluminescent specie is classified as non-bioluminescent (Summarized by Claes *et al*., 2024).

Yet, the results of Johnsen *et al*. (2014) and Krohn-Pettersen (2023) on flash kinetics are important because they front new methods in taxa recognition that aid the understanding of the marine bioluminescent community, which can help preservation of biodiversity.

1.4 Bioluminescence in zooplankton

Most of the bioluminescence observed at the sea surface during night is produced by dinoflagellates (a phytoplankton class, comprising autotrophic, mixotrophic and heterotrophic taxa) and zooplankton (Swift *et al*., 1983). Zooplankton are small aquatic heterotroph primary or secondary consumers,

feeding on phytoplankton, smaller zooplankton, or other organic matter (Reviewed by UiO, 2022). Their size also greatly varies from picozooplankton under 2 µm, to nanozooplankton (2-20 µm), microzooplankton (20-200 µm), and up to mesozooplankton (0.2-20 mm) and lager macrozooplankton and megazooplankton up to several meters (Sieburth *et al*., 1978). According to the Census of Marine Zooplankton project (Cmarz.org, 2008), there are five phyla in the protozoan kingdom and 11 phyla in the animal kingdom that contain zooplankton. They can be divided into two main categories; holoplankton that spend their whole lives as plankton in the water column, and meroplankton which are only planktonic at certain stages of their lives (Bertolo, 2022). Zooplankton are ecologically important because they transfer energy between phytoplankton and larger predators (Lomartire *et al*., 2021; UiO, 2022), and take part in the biogeochemical cycling of the biological pump (UiO, 2022). They can also be considered as early indicators for climate change, changes in temperatures and distinct water masses. This is because, based on definition, they cannot swim against the ocean currents so they move with the currents and can thus shift their spatial range (Ratnarajah *et al*., 2023).

Eight of the 11 zooplankton phyla in the animal kingdom contain bioluminescent species (Claes *et al*., 2024; Cmarz.org, 2008). In most of the phyla containing zooplankton, under 5% of the species are bioluminescent, except for ctenophores, where 17% of their 120 species are luminescent (Claes *et al*., 2024). However, of the 2781 bioluminescent species Claes *et al*. (2024) estimated, 84% are from chordates, arthropods, and mollusks. Although most of the chordates are not zooplankton, the tunicates (a chordate subphylum containing zooplankton (Letendre *et al*., 2024; WoRMS, 2024)) contain around 20 bioluminescent species (Claes *et al*., 2024). Cnidarians and annelids contain roughly 300 bioluminescent species.

1.5 Zooplankton monitoring in the past and present

Zooplankton can disperse with the changing oceans, being an early warning signal of a changing ocean. However, this warning signal can only be detected if we know what species are present and their distribution ranges (Bucklin *et al*., 2021). In order to do this, accurate identification is central, yet demanding (Bucklin *et al*., 2021; Kaiser *et al*., 2011). Bucklin *et al*. (2021) summarizes the main challenges of accurately identifying species; (1) many species are morphologically identical, making it difficult to distinguish them, (2) many species are found in different oceans, leading to genetic divergence, and (3) the biodiversity of an area can have a high local to global ratio, meaning that there can be multiple species with a varying distribution range. Consequentially, they can disperse unnoticed to new regions and impact the local pelagic food webs, carbon cycling and ecosystem sustainability. This shows how zooplankton can become invasive species, posing a threat to ecosystems and biodiversity. To detect changes in biodiversity and to be able to prevent the consequences, it is crucial to accurately identify species.

Classical zooplankton biodiversity monitoring uses trawls and nets of various sizes, followed by morphological identification. This is for example done in the Barents Sea, one of the most productive oceans in the world. The Norwegian Institute of Marine Research (IMR) and Knipovich Polar Research Institute of Marine Fisheries and Oceanography (PIRNO) have yearly cruises to assess plankton biodiversity (Eriksen & Meeren, 2021). The results from the cruise are used in international and national reports and publications, like the Oslo-Paris Convention (OSPAR) and Intergovernmental

Science-Policy Platform on Biodiversity and Ecosystem Services (IPBES), as well as regulating local fish stocks. This cruise has been conducted yearly during the last 20 years (Eriksen & Meeren, 2021).

This example of plankton monitoring is crucial for biodiversity knowledge and understanding how to manage the oceans. However, sampling done by nets can be quite time consuming and not all species are well suited for this type of collection, like the coelenterates which are easily damaged (Haddock & Case, 1999). In addition, samples from net tows are typically morphologically and molecularly identified, which is both costly, and requires unique researcher knowledge (Lindeque *et al*., 2006).

Due to climate change and increased human activity, like the light pollution seen in the Arctic or coastal darkening in the Norwegian fjords, there can be changes in biodiversity and zooplankton distribution ranges, stressing a need for increased monitoring. Simultaneously, there is an urge to reduce time and costs spent on monitoring, as well as increase the accuracy to identify species. This can be particularly important to detect cryptic, rare, and newly introduced species (Bucklin *et al*., 2021; Lindeque *et al*., 2013). Therefore, various methods are being tested as new ways to monitor zooplankton. One emerging and successful technique is environmental DNA (eDNA) metabarcoding. Because organisms shed DNA into their habitat, this can be collected and analyzed molecularly, identifying the DNA present in a sample (Taberlet *et al*., 2012). The advantage of this method is that the specimens do not need to be captured (Taberlet *et al*., 2012) and the sampling method is not limited by the bias of a net (Djurhuus *et al*., 2018). Djurhuus *et al*. (2018) compared eDNA metabarcoding with morphological identification of zooplankton collected by a plankton net, and concludes it is a sufficient method. It also detected a few rare species that were originally not morphologically identified, leading to fewer human errors. One limitation they addressed was DNA degradation, affecting the sequence success. In addition, DNA of distant species drifting with currents will provide false information on taxa present and different species' rates of DNA shedding can alter the perspectives on species composition (Djurhuus *et al*., 2018). Furthermore, eDNA, as any DNA based techniques, is dependent on good-quality reference library and at the current state this is very limited for the certain zooplankton taxa (Bucklin *et al*., 2021; MZGdb, 2024). For example, the Metazoo Gene database (MZGdb, 2024) shows that 30% of the 66809 invertebrate species have a reference COI DNA sequence, in contrast to the vertebrates where 67% of the 13773 species have a reference COI sequence. To reduce incorrect information, several methods should be used simultaneously to gather holistic data on biodiversity, and due to the high diversity in zooplankton, not all taxa are identifiable using one method (Djurhuus *et al*., 2018; Lindeque *et al*., 2013). With a changing climate resulting in changing seas, discovering, and monitoring species has become a central focus. We cannot protect what we are not aware of, and learning about different species and their dynamics will help to conserve them.

Exploring the field of zooplankton bioluminescence can help understand their taxonomy, dynamics, abundance, and distribution. As stated above, bioluminescence has attempted to be used as a tool to identify different zooplankton taxa. In Kongsfjorden, Svalbard, Johnsen *et al*. (2014) showed that 80% of bioluminescent flashes recorded from a bathyphotometer measuring flash kinetics were taxonomically identified. A similar study was recently performed by Krohn-Pettersen (2023) concluding that flash kinetics had llimited potential for *in situ* taxa recognition with the techniques used. However, Krohn-Pettersen does stress that there is potential for flash kinetics to be used in monitoring zooplankton because some taxa were classified in laboratory conditions.

Although flash kinetics may be a useful tool, there are some limitations. Firstly, a bathyphotometer, the device used to measure flash kinetics, is a rather invasive method, destroying the specimens. Secondly, both Johnsen *et al*. (2014) and Krohn-Pettersen (2023) showed that the bathyphotometer failed to record a viable signal from some small specimens, like the bioluminescent euphausiid *Thysanoessa* sp. that are numerous in Kongsfjorden (Berge *et al*., 2012). Other studies have been able to measure *Thysanoessa* sp. as well as other euphausiids, but mention that a bathyphotometer provides limited information on this taxa, due to their counterilluminating properties and small size, so mechanical stimulation is preferred to increase accuracy (Cronin *et al*., 2016). These results and limitations show the potential, as well as need, for further research and advancing methods that can measure bioluminescence.

Therefore, measuring spectra of zooplankton could be one solution, solving some of these challenges. Spectral measurements are less invasive, and normally do not damage the specimens. It also allows for mechanical stimulation, which is comparable to how bioluminescence is stimulated in nature (Haddock & Case, 1999). Measurements can also be detected from organisms that are too large or too small for the bathyphotometer, and with different bioluminescent intensities. Multiple spectral measurements have been performed in various locations, showing that there is variation in spectral properties between species (Gouveneaux *et al*., 2017; Haddock & Case, 1999; Herring, 1983; Latz *et al*., 1988), but none of the respective studies have done experiments aiming to develop a method that can allow for taxa recognition based on spectral bioluminescence.

1.6 Study aim

This thesis is part of a research project called Light as a Cue for life in Arctic and Northern Seas (LightLife), funded by the Research Council of Norway (2021-2024). The purpose is to investigate how changing light climate affects zooplankton. There are three closely correlated work packages (WP), and this research is a part of WP3: "Bioluminescence (BL) as contributing factor to underwater light environment in the Arctic." One central aim of WP3 is to develop new techniques to study bioluminescent zooplankton and create a bioluminescent reference database for potential species identification.

The aim of this thesis is to develop an improved method for *in situ* taxa identification of Arctic and Northern zooplankton, based on their spectral bioluminescence. To achieve this, various bioluminescent zooplankton will be collected and stimulated to produce a bioluminescent light that will be recorded with a spectrometer. Different methods will be used to stimulate bioluminescence, and the most efficient for each taxon will be assessed. Three aspects of the bioluminescence emission spectra for each taxon will be compared: the λ_{max}, FWHM and the shape of their bioluminescence spectra, denoted as spectral shape and spectral curve. The latter includes characteristic features not detected by the spectral parameters (λ_{max} and FWHM). The λ_{max} and FWHM will be included in the WP3 reference database. The focus will also be to investigate if there is variation in spectral composition between different taxa and between different scales; month and location, as well as method if several are used on one taxon. The results will then be discussed, evaluating to what extent spectral composition can be used for *in situ* zooplankton taxa identification.

2 Methods

2.1 Study sites

To compare variation in bioluminescence, opportunistic fieldwork was conducted in Kongsfjord, Svalbard, during the polar night 2023, at various locations of the Trondheimsfjord, mid-Norway, during summer and fall 2023, as well as in Hopavågen during October, a bay located just outside the mouth of the Trondheimsfjord. The locations lie in the Norwegian Sea – Greenland Sea basin and receive warm water from the North Atlantic Current (NAC) (Figure 2). The NAC together with cold Arctic currents defines the physical properties, like temperature, and biotic factors of the area (Hamre, 1994).

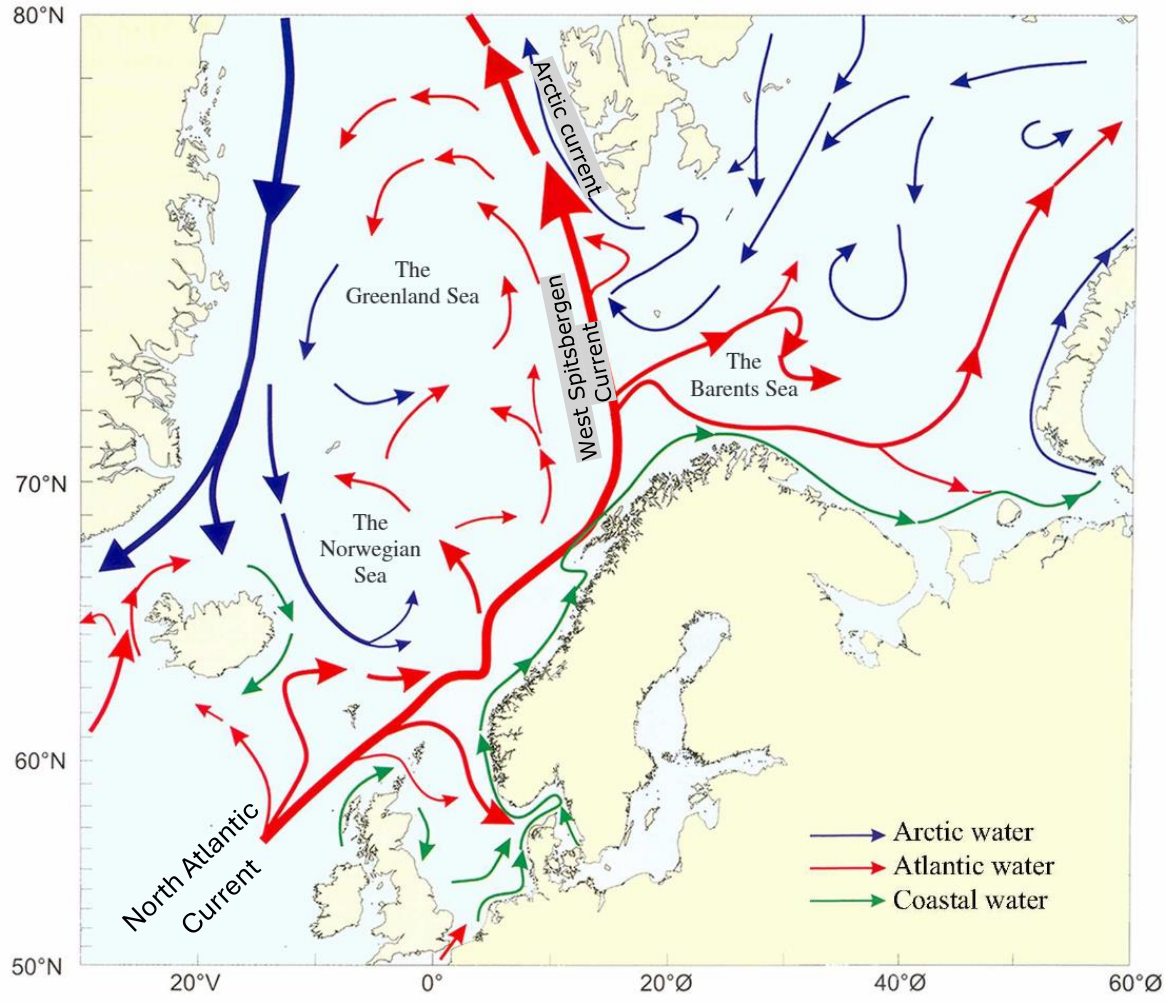

Figure *2*: Currents in the Nordic Sea region. Blue arrows are Arctic waters, orange arrows are the Atlantic waters, and green arrows are the Coastal waters. Modified from the Norwegian Marine Research Institute.

Kongsfjorden is located above the polar circle, at 79°C North, 12°C East (Grenvald *et al*., 2016), on the West side of Svalbard (Spitsbergen). The fjord is an open glacial fjord that faces the Greenland Sea, and receives warm water from the West Spitsbergen Current, and cold water from the Arctic Current (Cottier *et al*., 2005; Hop & Wiencke, 2019). Samples were collected in January when the average atmospheric temperature is -15°C (Svendsen *et al*., 2002), while the sea surface temperature is around 1°C and has a salinity fluctuating around 34,8 (Grenvald *et al*., 2016). The downwelling spectral irradiance during midday in January, has a maximum transmission of 455 nm, in the blue-green color

spectrum (Cronin *et al*., 2016). Regarding the fjords biodiversity, long-term monitoring of zooplankton has shown population increases, hence there is grounds to believe that they benefit from Atlantification of Arctic waters (Hop & Wiencke, 2019), meaning that the Artic ocean is slowly becoming more similar to the Atlantic ocean in terms of biotic and abiotic factors.

The Trondheimsfjord is located 63°C North, 10°C East, opening out into the Norwegian Sea. According to Copernicus Marine MyOcean Viewer, the sea surface temperature during the field periods ranges from 11 – 15°C. Long term monitoring from 1963-2014 in the deepest areas of the Trondheimsfjord has shown an increase in temperature, by roughly 0,5 – 2°C, depending on the area. During winter, the green color wavelengths travel the furthest in the fjord, down to roughly 40 m (Sakshaug *et al*., 2009). Zooplankton are monitored using acoustics, together with other biotic factors from a permanently deployed buoy. A recent report on the health status of the Trondheimsfjord has stated that zooplankton abundances show similar trends today as in the 1960's, but the species composition has potentially changed towards more southern specimens (Trøndelag Fylkeskommune , 2023).

Hopavågen is a small bay and has a narrow channel called Straumen, connecting it to the mouth of the Trondheimsfjord. It is 31 m at its deepest (Marion, 1966). The tidal inflow and outflow of the channel is delayed compared to the rest of the fjord, due to its narrow properties. This is believed to affect the biology, like how its littoral zone has fewer specimens compared to other similar bays (Marion, 1966). Outside Straumen lies Stavøya. Measurements on the light climate by Stavøya were done by an NTNU course, and they found that green wavelengths (500-550 nm) remain the longest in the water column (unpublished data, NTNU Enabelling Technologies course BI3070 2023).

2.2 Sampling

Zooplankton was collected opportunistically using several methods (Table 1), and in multiple locations (Figure 3). In the Kongsfjord and Trondheimsfjord species were collected with oblique tow from 0 to 200 m and 0 to 500 m depths, respectively (Figure 4A). 100 µm and 200 µm plankton nets with non-filtering cod end were pulled vertically from research vessels at a slow speed to best preserve the specimens. The cod end was carefully emptied into a bucket, and the sample was diluted with cold filtered seawater before a light tight lid was placed on the bucket.

In addition, surface dwelling species were collected using handheld methods in Kongsfjord, Trondheimsfjord and Hopavågen (Figure 4B). This was done using custom-made handheld containers and handheld nets, for instance, the bucket on stick-approach. Larger ctenophores (> 3 cm long) were more vulnerable and prone to damage, so they were only successfully retrieved by gentle methods.

In the lab, zooplankton samples were carefully sorted with spoons and pipettes, depending on their size, and preliminary species identification was done to the lowest taxonomic level possible. Specimens with minimal damage were used in the measurements, but due to limited numbers of some taxa, the condition was rather noted (i.e. damaged or whole). During all handling of the specimens, red light was used because this is associated with a gentler working light for zooplankton, compared to white light (unpublished data by Majaneva and Viljanen). After sorting, they were stored in a dark room at a temperature corresponding to their natural habitat for dark and temperature acclimation.

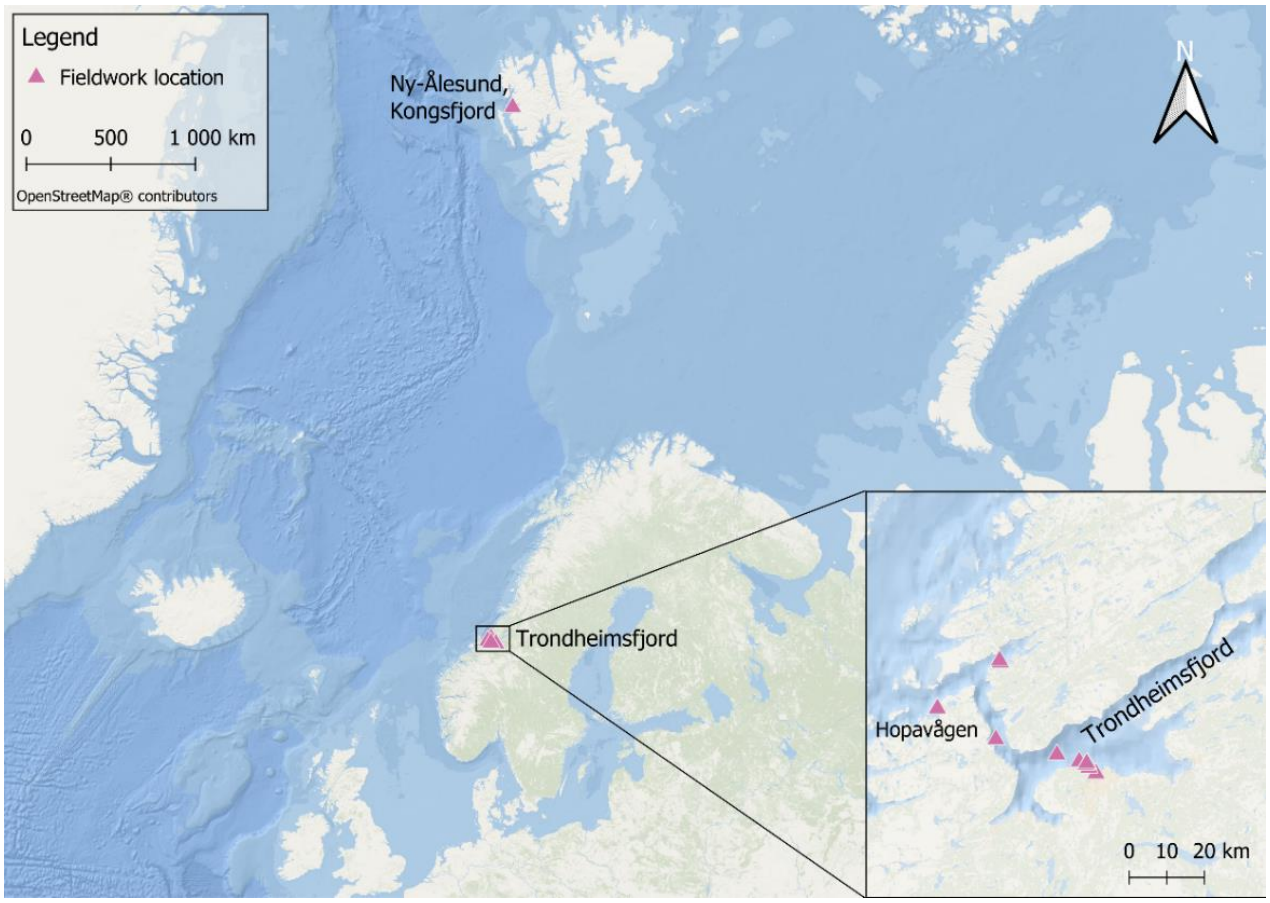

Figure 3: Map of fieldwork locations. The triangles are the sampling locations in the Trondheimsfjord, Hopavågen and Ny-Ålesund. In the bottom right corner, there is a zoomed view of the Trondheimsfjord with all sampling locations. Map from Open Street Map contributors. Created by Hedda Førde in QGIS Desktop 3.30.2.

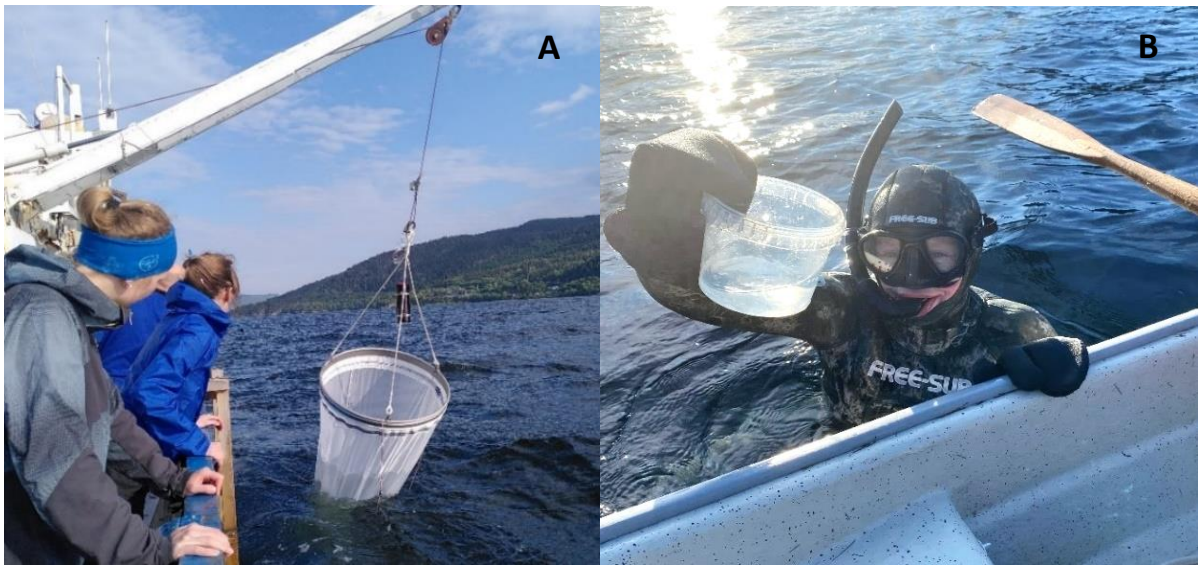

Figure 4: Different techniques to collect zooplankton. A: collecting specimens with a plankton net from RV Harry Brothen. Photo by Martta Viljanen. B: collecting specimens using handheld containers was done from both land and water, as seen here. Photo by Maria Rosland.

Table 1: Overview of the fieldwork conducted, sampling methods, taxa (classified to the lowest confident taxonomic level) found at various locations and the method used to stimulate bioluminescence.

LOCATION	MONTH	SAMPLING METHOD	SPECIMENS CAUGHT	BIOLUMINESCENCE STIMULATION METHOD
Ny-Ålesund, Kongsfjord	January	200 µm plankton net. Sampling at 0-200 m depth	Euphausiid indt., *Hansarsia megalops*	Mechanical
			Euphausiid indt., *Hansarsia megalops*	Light
		Handheld containers. Sampling at the water surface	*Beroe* spp., *Bolinopsis* spp., euphausiid indt., *Euplokamis* cf. *dunlapae*, *Hansarsia megalops, Mertensia ovum*	Mechanical
			Euphausiid indt.	Light
Ila, Trondheimsfjord	June	Handheld containers. Sampling at the water surface	*Aurelia aurita, Beroe* spp., *Bolinopsis infundibulum*	Mechanical
Trolla, Trondheimsfjord	June	200 µm plankton net. Sampling at 0-300 m, 0-370 m, 0- 400 m, and 0-420 m depth	*Aetideopsis armata*, euphausiid indt., *Metridia* spp., *Tomopteris* spp., *Thysanoessa raschii*	Mechanical
Various locations in the Trondheimsfjord	August	200 µm plankton net. Sampling at 0-300 m, 0-525 m, 0-500 m, and 0-100 m depth	*Beroe* spp., *Clythia* spp., ctenophore indt., coelenterate indt., hydrozoan indt., *Leuckartiara octona*, *Metridia* spp., *Modeeria rotunda*, siphonophore indt.	Mechanical
			Beroe spp., *Clythia* spp., hydrozoan indt., *Leuckartiara octona, Metridia* spp., siphonophore indt.	Osmotic stress
Various locations in the Trondheimsfjord	October	100 µm plankton net at 500 m depth	Euphausiid indt., *Metridia* spp., *Tomopteris* spp.	Mechanical
			Metridia spp., *Tomopteris* spp.	Osmotic stress
			Tomopteris spp.	Light
Hopavågen	October	Handheld 200 µm plankton net at 0-30 m	*Bolinopsis infundibulum, Clytia* spp., coelenterate indt., lobate indt., *Tomopteris* spp.	Mechanical stimuli
		Handheld containers. Samples collected at surface	*Clytia* spp., coelenterate indt.	Osmotic stress

2.3 Bioluminescence measurements

2.3.1 Set up

To measure spectral bioluminescence the spectrometer QE Pro (Ocean Insight) with an optical fiber attached was used (Figure 5). The sensor measures light radiance. To measure the bioluminescent signals, the fiber was placed close to the organism that was stimulated to flash or glow (see section 2.3.2, stimulating bioluminescence). Note that the terms “flash” and “glow” will be used interchangeably.

To read the signal, the QE Pro was connected to a computer, and the program OceanView 2.0.8 (OceanInsight, 2024) displayed and saved signals from the sensor on a wavelength (X) – intensity (Y) graph (Figure 6). To remove background noise a dark reference spectrum was measured, updated, and applied regularly using the live dark capture button. One measurement was set to last from 20 – 60 seconds depending on how fast the taxa started to bioluminess. The integration time was set to 0,5 – two seconds (see section 3.2 for taxa specific integration times), depending on the intensity of the flash. This means, if one measurement lasted 60 seconds and the integration time was set to two seconds, 30 spectra would be recorded.

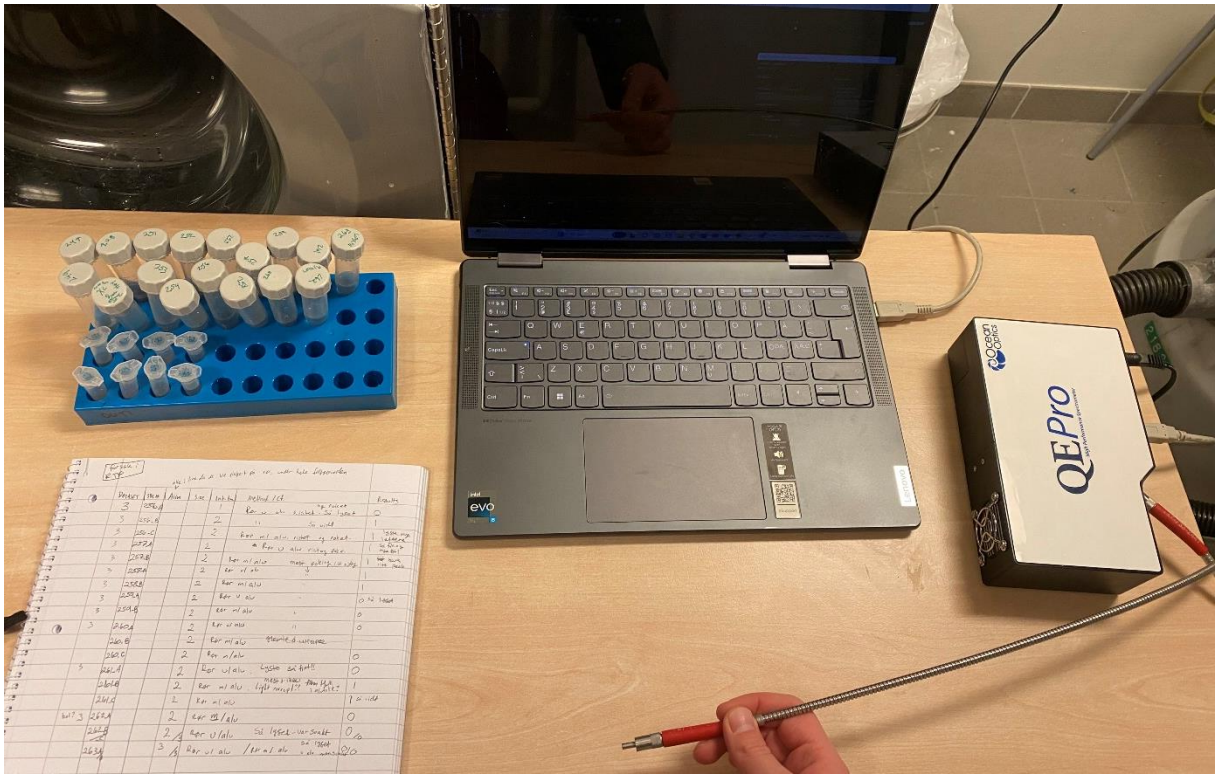

Figure 5: experimental set up. PC displaying OceanView, QE Pro connected to PC and optic fiber attached. The tubes to the left are one set of live specimens that have been measured and labelled SBL###. Figure 6 displays the PC screen during a bioluminescent measurement. Photo: Hedda Førde.

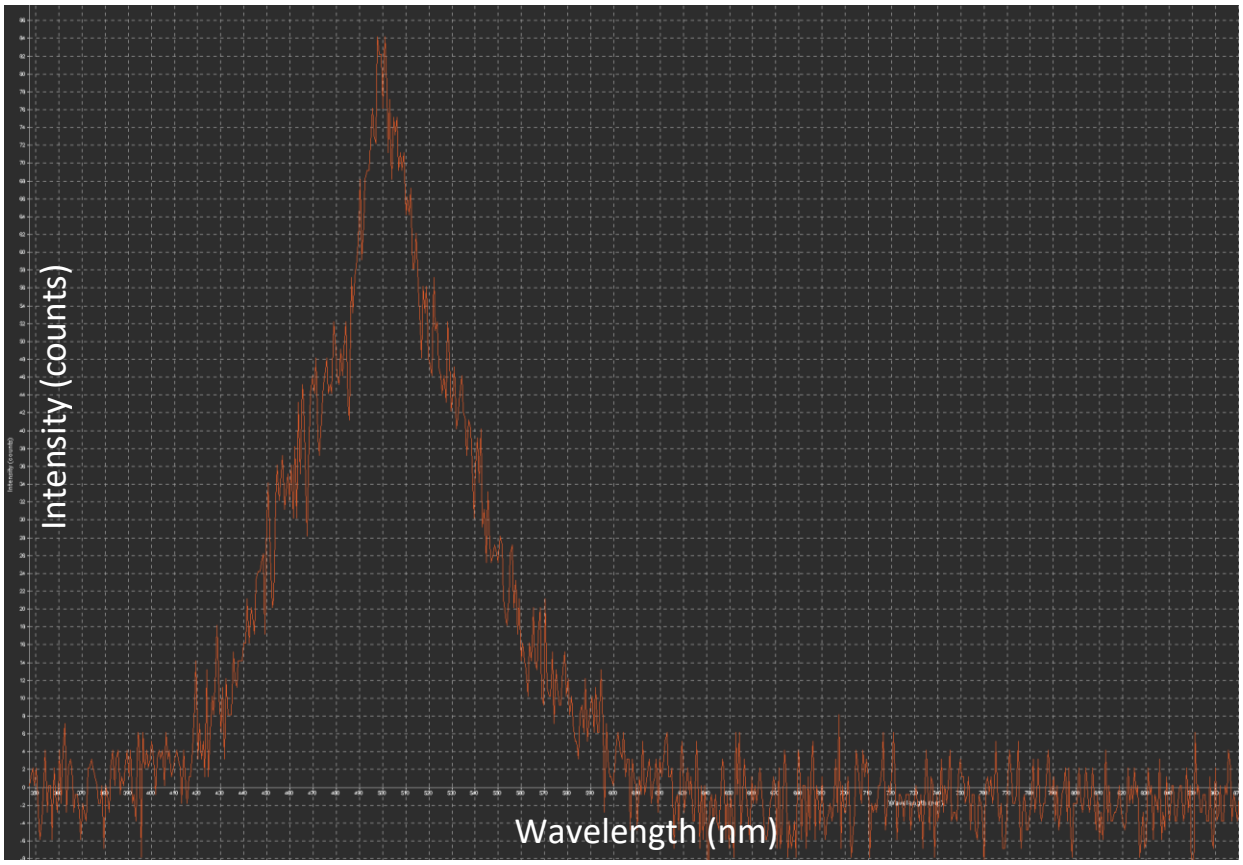

Figure 6: screenshot from a spectral recording in OceanView data program. For each measurement, multiple spectral recordings were done. One recording becomes one file. The number of recordings depends on recording time and integration time. If the recording time (i.e. duration of one measurement) was 50 seconds, and the integration time was 1 second, a measurement would be recorded every second for 50 seconds, giving 50 spectral recordings, like this one. Every recording is different because the bioluminescent flash is inconsistent. Photo: screenshot by Hedda Førde from OceanView 2.0.8.

2.3.2 Stimulating bioluminescence

The stimulation and measuring were done in a dark and cold temperature-controlled laboratory, only with a red working light. Measurements were completed in filtered seawater baths placed on ice, to minimize temperature variation. Using filtered seawater was important so that no small luminescent organisms like dinoflagellates would be present (Bowlby *et al*., 1991). A thermometer was placed in the water bath from the start of the experiment, to check for temperature variation. Start and end temperatures were noted, but the thermometer was checked regularly to detect when temperature was changing.

One set of living specimens, with 5 to 20 individuals (See Figure 5 for an example of a set), was completed in one round of measurements to minimize temperature and light variation. Prior the measurements, the individuals in the set were placed into their own small measurement container (See Figure 5, and further details are described in section 2.3.3: Taxa specific methods) where the measurement would occur, and then given an ID in the format SBL### (i.e. SBL001). Depending on the size of the specimen, pipettes and spoons were used to place them in their respective containers. Gentle movements were important to not shock and accidentally stimulate bioluminescent flashes. Since the sensor requires high proximity to the specimens, it was important for the container to be as small as possible to constrain the area where the organism could freely move (Figure 7).

The last preparation was to set up the QE Pro as shown in in Figure 5, and the red working light was switched off. If there were any dim light sources in the room (i.e. curtains did not block out all sunlight) visible in OceanView, either the source or the measurement container was covered (Figure 7).

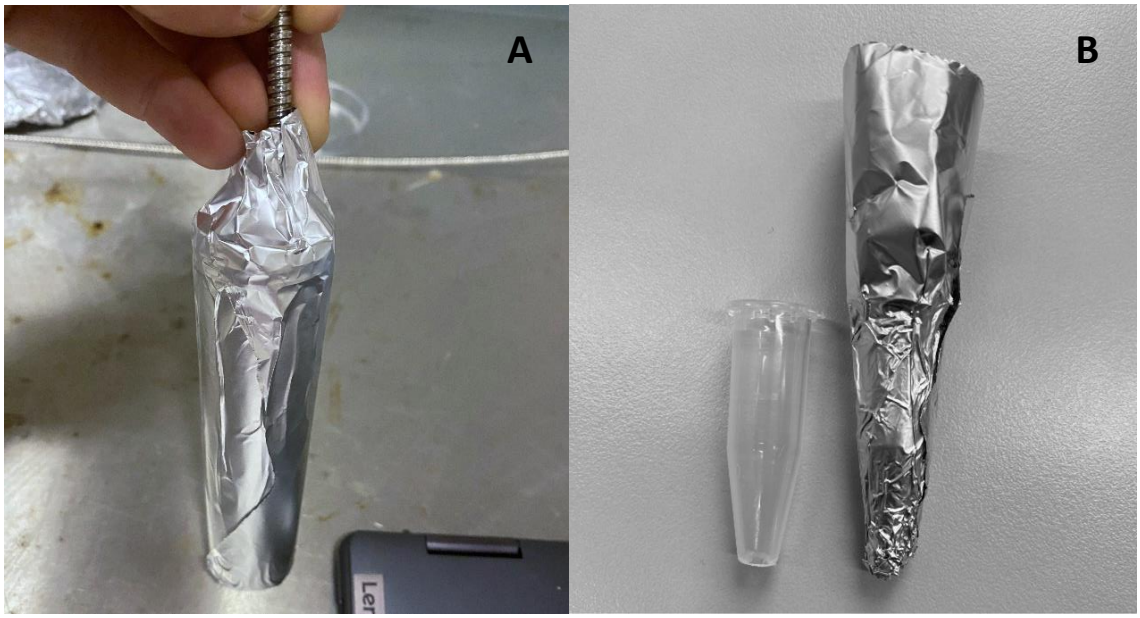

Figure 7: example of two different containers used to stimulate bioluminescence. **A**: 50 ml Falcon tube with fiber inside to measure the spectral emission. The tube and fiber are covered to create a light proof environment. A live specimen is placed inside, and turbulence is created by shaking the tube to stimulate bioluminescence. Photo by Hedda Førde. **B**: Example of 1,5 ml Eppendorf tube with cut end. The tube without aluminum foil (**B**, left) is identical to the tube covered in foil (**B**, right). The covered tube has excess foil to wrap around the fiber, which is inserted from the top, blocking out ambient light (same as **A**). Small holes are made in the bottom of the aluminum foil so water can move in and out of the tube. During the measurements, the Eppendorf tubes with live specimens are placed on a rack in cold sea water. To stimulate bioluminescence, the tube is lifted out of the water. If necessary, mechanical stimulation is applied by tapping the tube on the table. Photo by Hedda Førde

Three stimulation methods were used: mechanical, light and osmotic stress. Electricity and KCl has also been proven successful stimulation methods in other studies (Buskey & Swift, 1985; Haddock & Case, 1999; Latz *et al*., 1988), but for practical reasons, they were not used. To mechanically stimulate a specimen, either the container was shaken, a rod for poking was used, or the specimen was lifted out of the water using a small sieve. A dimmable headlamp with white light was used for light stimuli. The final method used was osmotic stress. This was done by placing them in freshwater to create hypotonic cells as in Chen *et al*. (2007), or in soap water to weaken cell structures, where inspiration was taken from Gouveneaux *et al*. (2017) whom used surfactants to stimulate bioluminescence in *Tomopteris* spp. and Heerklotz and Seelig (2000) who described how detergents (detergent is a type of surfactant) affect cell membranes.

In some cases, several methods were tested on the same individual, but over several recordings. The treatment or method was noted using the name SBL###_x (for example, SBL001 for the first treatment, and SBL001_B for the next treatment on individual 001). Mechanical stimulation was the initial method used for all specimens because it is similar to how bioluminescence is stimulated *in situ*. Several methods were used if no flashes were recorded. Often bioluminescence was observed from a specimen, but the glow or flash was too dim or sudden to record. In these cases, aluminum foil was used to enhance capture of the signal, and/or the integration time was adjusted to a longer duration.

For every trial, the following information was noted: taxonomic ID, SBL ID, living condition (active, passive, damaged etc.), size (millimeters), integration time (seconds), stimulation method, confounding variables, and immediate results (1 or 0). Every file was saved with time stamps, so if an individual was given an incorrect name, (i.e. spelling mistakes, forgetting to change name between individuals or test) the file could easily be corrected by looking at the time stamps of the saved files.

One recording was initiated, and the bioluminescence was stimulated with one of the stimulation methods and measured with the fiber simultaneously (Figure *5*). After the measurements, specimens were morphologically identified and preserved on 96% EtOH in their own Eppendorf tube, labelled with their ID.

2.3.3 Taxa specific methods

To improve the quality of the measurements, each taxon was treated slightly differently, described in the following sections. The methods used for stimuli and container design are based on preliminary tests, unpublished data, and other studies working on the same or similar taxa. Common for all containers was that they had to be made lightproof and they should be able to reflect light in case the glow became too dim.

2.3.3.1 Coelenterates

The following criteria were made for the coelenterate measurement containers:

- Suitable opening at the top for the spectrometer fiber and specimen to enter,
- Enough space for specimen, but not too small so its bioluminescence is stimulated.

This was central to the coelenterates because the preliminary trials showed that they did not flash if the space was too constrained. Furthermore, the flash often spread over the specimens' body, so it was difficult to measure without the help of aluminums' reflective properties.

Examples of containers were plastic cups or tubes of various sizes (50 ml, 15 ml and 5 ml tubes were used) (Figure 7). The stimulation method used were mechanical and osmotic stress (Table *1*).

2.3.3.2 Metridia spp.

Metridia spp. is known to glow by mechanical stimuli and when taken out of the sea water (Unpublished data) thus a container adapted for both mechanical stimuli and air exposure was needed. The requirements for these containers were:

- Narrow opening at the top for the spectrometer sensor,
- Fine mesh or small holes in the bottom for water to seep in and out of the container, without specimen escaping.

One example of a suitable container is 1,5 ml Eppendorf tube with a cut end (Figure 7). Aluminum was wrapped around the tube, and small holes were made through the foil using a needle. After a specimen was inside the tube, the fiber was placed in the opening of the container. The Eppendorf tube was then lifted out of the water, and the recording was initiated. Bioluminescence was stimulated with mechanical stimuli (shaking the tube) or osmotic stress (dipping tube into the soap or freshwater solution) (Table 1).

2.3.3.3 Euphausiid

The same Eppendorf tubes and stimulation method was used for small euphausiid (<0,5 cm), as for *Metridia* spp*. (see Metridia* spp.*)* For larger euphausiid (>0,5 cm), the specimens were placed on Petri dishes, and they were stimulated mechanically or by light (Table 1). During light stimuli, euphausiid were placed in a room with a dim light for 15 minutes to 3 hours. Due to euphausiid's counterilluminating properties, the photophores would in theory light up over time when exposed to dim light (Haddock *et al*., 2010). When this was observed, the light was switched off and luminescence was measured.

2.3.3.4 Tomopteris spp.

The containers and mechanical stimulation method used were the same as euphausiid and *Metridia* spp., in addition to a small sieve for air exposure and osmotic stress. Stimuli using light stimulation was done by quickly flashing a white light from a headlamp onto the organism. Inspiration was taken from Gouveneaux *et al*. (2017) and Gouveneaux and Mallefet (2013).

2.4 Species identification

2.4.1 Morphological species identification

After the measurements, organisms were re-identified to the lowest taxonomic level feasible. Thorough identification was done after measurements to minimize handling time prior to the measurements. A light microscope and various identification keys were used (Castellani & Edwards, 2017; Johansen *et al*., 2021).

2.4.2 Molecular species identification

Specimens for molecular identification were chosen based on any uncertainties during morphological identification, and individuals with typical and atypical spectral composition to secure correct species identity. In total 75 specimens presenting 14 taxon were selected and the DNA was extracted with a modified Chelex rapid boiling procedure (Granhag *et al*., 2012) for coelenterates and with Qiagen DNeasy Blood & Tissue Kit for crustaceans. In both extraction methods roughly 0,5 mg of body tissue was pipetted into a 1,5 mL Eppendorf tube, and the lid was left open for 12 hours to allow the EtOH to evaporate. In Chelex DNA extraction, 30 µL of 6% Chelex® 100 resin (BioRad) in 50 mM Tris, pH 8.0, and 0.5 mM EDTA was added to the Eppendorf tube. It was then heated to 98°C for 10 minutes, before centrifuging 10 minutes at 4°C, with 15000 RPM. The DNA, supernatant, was transferred to a new 1,5 mL Eppendorf tube, and frozen (-20°C). Qiagen DNeasy Blood & Tissue Kit was used according to the manufacturers protocol (QUIAGEN Group, 2016).

Polymerase chain reaction (PCR) was then used to amplify specific target regions depending on the taxa (Bucklin *et al*., 2021; MZGdb, 2024). For crustaceans, the mitochondrially encoded cytochrome c oxidase I (mtCOI) was chosen and universal Folmer primers (LCO1490 and HC02198; Folmer *et al*. (1994) was used. A PCR mix of 20 µL contained: 1 µL Folmer LCO, 1 µL Folmer HCO, 4 µL Phire® reaction buffer, 0,4 µL dNTP, 0,6 µL DMSO, 11,6 µL nuclease free water, 0,4 µL Phire® Hot Start DNA polymerase, and 1 µL specimen DNA template. The mix was pipetted into PCR strips that were placed in a PCR machine (SimpliAmp Thermal Cycler, Applied Biosystems by Life Technologies). For ctenophores, universal eukaryotic primers for and 18S (Kober and Nichols 2007) were used, and for hydrozoan mitochondrial ribosomal RNA (16S) was amplified using the primers SHA and SHB (Cunningham & Buss, 1993). Due to low success rate with *Metridia* spp. additional zooplankton specific primers Zplank F1 t1/ Zplank R1 t1 (Prosser *et al*., 2013) were used. For more details about the primers and PCR cycle, see Table 2.

Table 2: The different primers used for different taxa. The PCR cycle with 1: denaturing/ initial phase, 2: annealing, and 3: elongation.

PRIMER	TARGET SPECIES	TARGET REGION	PCR CYCLE
18SF/18SR	Ctenophore	18S	1: 98 °C – 5 min, 2: (98 °C – 8 sec, 64 °C - 10 sec, 72 °C – 1 min) x 35, 3: 72 °C – 5 min, 4 °C – 10 min
LCO/HCO	Euphausiid, *Metridia* spp., *Tomopteris* spp.	CO1	1: 98 °C – 5 min, 2: (98 °C – 8 sec, 57 °C - 10 sec, 72 °C – 1 min) x 40, 3: 72 °C – 5 min, 4 °C – 10 min
SHA/ SHB	Hydrozoa, *Tomopteris* spp.	16S	1: 94 °C – 5 min, 2: (94 °C – 20 sec, 50 °C – 45 sec, 68 °C – 2 min) x 35, 3: 68 °C – 5 min, 4 °C – 10 min
Zplank F1 t1/ Zplank R1 t1	*Metridia* spp.		1: 98 °C – 5 min, 2: (98 °C – 5 sec, 50 °C – 45 sec, 72 °C – 20 sec) x 40, 3: 72 °C – 2 min, 4 °C – 10 min

Gel electrophoresis was used to confirm DNA presence in the PCR product. An 1,5% agaros gel was made and placed in 1/50x TAE buffer in the electrophoresis cage. The PCR product was sampled into the gel. Successful products showed a clear band on the gel under UV light (Figure 8). These were stored in the -20C freezer.

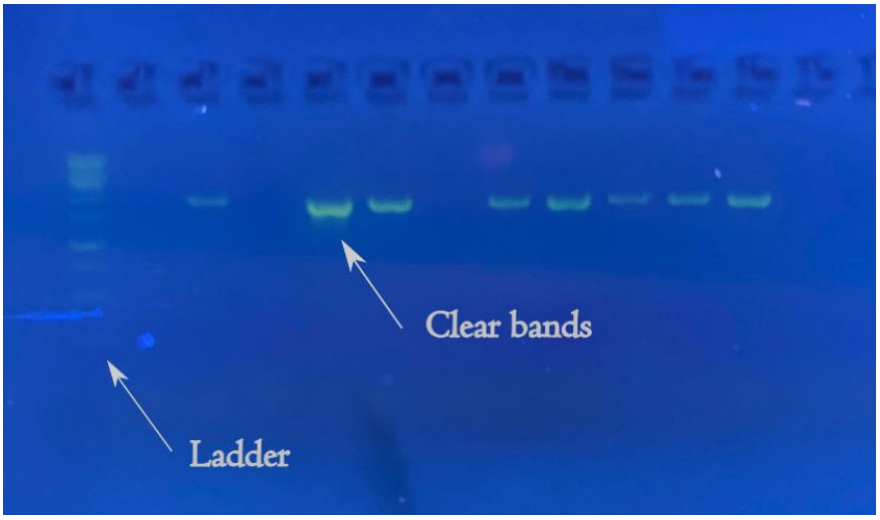

Figure 8: example bands of PCR product (light, signle, stripes) showing a successful DNA extraction and PCR. The ladder is to see the spatial range for where bands should lie. Photo by Hedda Førde

The successful PCR products were sent to MacroGren Sequencing Service (MacroGen Inc, Amsterdam, Netherlands) for PCR product purification and sequencing. Sequencing was preformed using the Sanger method (Sanger *et al*., 1977). Due to time limitation, only forward direction sequencing was conducted. When the sequence chromatogram files were received, they were viewed in Chromas 2.6.6 (*Technelysium DNA sequencing software*, 2024) for cleaning and trimming the DNA sequence. Sequences were trimmed and cleaned as far as possible, or removed if the quality was too poor. A good sequence had nucleotide bases with high certainty (scores were given in the program), and low certainty for poor quality sequences. The sequences were saved as FASTA files, which were uploaded to National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) (NCBI, 1988-present). BLAST compares the uploaded biological sequence to a sequence database and calculates statistical significance of the matches. To assess occurrence of species present in the BLAST results, taxon name was searched for in the databases at Artsdatabanken and Global Biodiversity Information Facility (GBIF).

2.5 Data analysis

2.5.1 Extraction of parameters

A MATLAB code was used to extract the necessary spectral parameters from all files: λ_{max} (wavelength in nm at maximum intensity) and full-width-half-maximum (FWHM, the width of the curve in nm at half of λ_{max}). Peaks were smoothed with a moving average filter, with a width corresponding to 23nm.

Since multiple spectral recordings were obtained for each measurement, the was chosen to limit sampling bias of species with a constant glow or a repeating flash. The best signal was judged based on calculating signal-to-noise ratio (SNR) and visual inspection. First, the minimum peak limit was set to 1,0 x10^{-5} µmol photons. Then, the SNR was calculated by finding the ratio of the maximum signal to the minimum signal. The spectrum with the highest SNR was chosen for final analysis. A threshold of SNR=2 (±0,1 nm) was set because it sorted out most of the spectra with bioluminescent peaks that were indistinguishable from noise. Noise and artefacts above SNR=2 was screened by visually inspecting spectra with a relatively small FWHM and/or a deviating λ_{max} (see example of an artefact in Figure 9). If visual inspection of the spectra confirmed high noise or revealed an artefact, the spectra with the second highest SNR (given that the SNR was above 2 and no abnormalities were detected) was chosen. The spectral range was set to 350-700 nm, the range of visible light (National Aeronautics and Space Administration, 2010). The selected signals were compiled to create a dataset for analysis.

Finally, MATLAB was used to create normalized spectra, where the maximal intensity was set to 1 unit. This visually allowed comparison of spectral parameters within and among taxa.

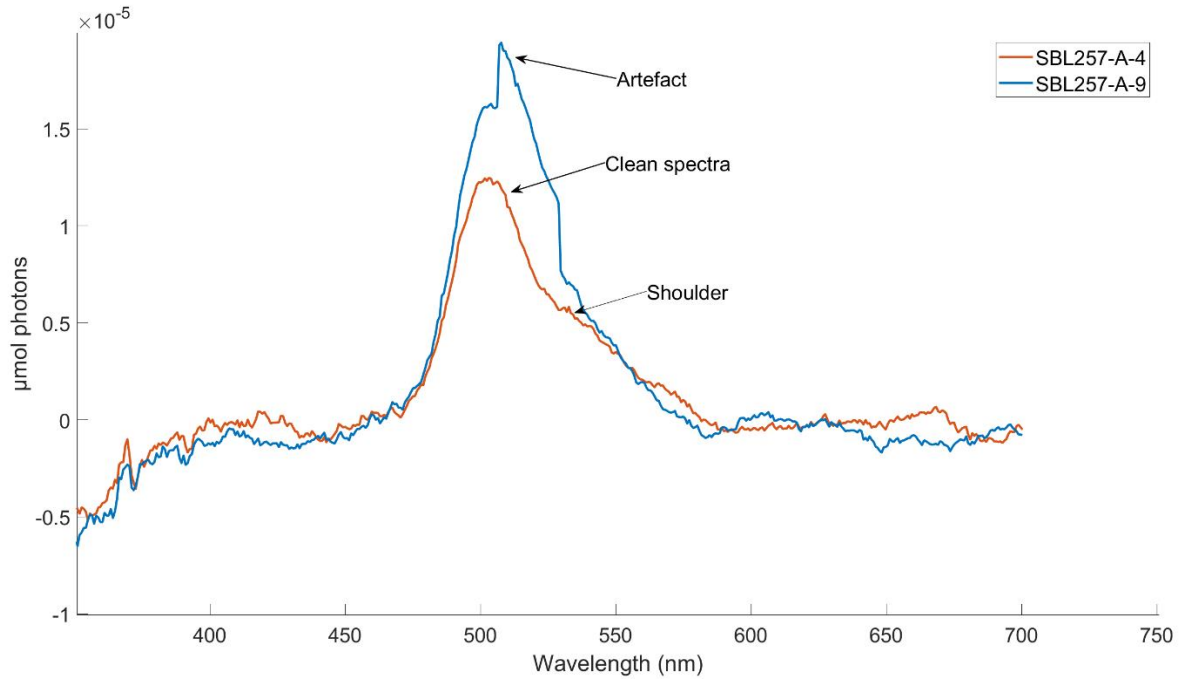

Figure 9: example of two recordings from the same specimen (*Clytia* sp., SBL257), during measurement A. The red line is a clean peak (SBL257_A_4) while the blue line (SBL257_A_9) contains an artefact (see arrow on figure). A shoulder is also seen around 540nm. SBL257_A_4 has an SNR=2,13, λ_{max}=503 and FWHM=44,8. SBL257_A_9 has an SNR=12,7, λ_{max}=508 and FWHM=39,8. If the SNR was solely considered, an artefact would be chosen for further analysis. However, due to further inspection of λ_{max} and FWHM, the spectra were visualized, resulting in the clean spectra, SBL257_A_4, being chosen instead.

2.5.2 Statistical analysis

The final dataset was analyzed in R studio (R version 4.3.0) (*Rstudio desktop*, 2024). Average λ_{max} and FWHM for each taxon and their respective standard error of means (SEM) were calculated. The SEM represents the uncertainty in the sample mean as an estimate of the population mean. It reflects how much the sample mean is expected to vary from the true population mean due to random sampling error.

Statistical analyses were performed to calculate the variation between the λ_{max} and FWHM within and among each taxa. To choose the correct statistical tests, independence, normality and distribution of residuals need to be checked. It is assumed that each datapoint is independent from another because most measurements were from different species. Normality and homogeneity was checked with QQplots as a visual representation, and the Shapiro-Wilk test was used as an additional confirmation to normality (Ford, 2015; King & Eckersley, 2019).

To examine the differences between groups, an Analysis of Variance (ANOVA) and Kruskal-Wallis test was used. ANOVA evaluates the differences between the means of more than two groups. It assumes that the data is normally distributed, that all observations are independent of each other, and that the variance level within each group is roughly equal. The Kruskal-Wallis is used when the assumption of normality is violated. However, the ANOVA and Kruskal Wallis test only claim if there is a statistically significant difference between variables; not which variables are different. Thus, if the tests show that there is a significant difference between groups, the Dunns test is used as a post hoc comparison. A Bonferroni adjustment to the p-value was done for the non-parametric data, which reduces the chance of performing a statistical type 1 error (Armstrong, 2014).

3 Results

3.1 Taxa recorded

In this study, 270 specimens were used for 390 completed measurements. 42 measurements were perfomed in Kongsfjorden, on various ctenophores, the euphausiid *Hansarsia megalops* and other euphausiid indt. 95 measurements were performed in June in the Trondheimsfjord, mainly on the copepod *Metridia* spp., but also on one specimen of the euphausiid *Thysanoessa raschii* and copepod *Aetideopsis armata.* 84 measurements were done in August, also in the Trondheimsfjord, mainly on *Metridia* spp., and several coelenterates. In October, two fieldwork periods were completed, one with nets in the Trondheimsfjord (104 measurements), where most of the annelid *Tomopteris* spp. were found, and one with handheld sampling methods in Hopavågen (66 measurements), where majority of the ctenophore *Bolinopsis infundibulum* and cnidarian *Clytia* spp. were found.

Measurements were completed on 21 different taxa, classified to the lowest confident taxonomic level (Table 3). Based on morphological identification, 16 taxa were identified, and the molecular identification confirmed the last five taxa to lower taxonomic levels. Of the 75 specimens prepared for molecular identification, 32 were sent to sequencing. Out of these 32 specimens, only 18 relevant and good sequences were retrieved. The good, but contaminated samples only showing bacteria or fungi were not considered relevant to include in this thesis.

The 18 good sequences confirmed two bioluminescent outliers, where one *Metridia* spp. accidentally had been classified as an euphausiid indt. and the second, reclassified a *Metridia* spp. to the copepod *A. armata*. Furthermore, molecular results also confirmed some of the euphausiids to species level; *H. megalops* and *T. raschii*. One ctenophore indt. was classified to a cnidarian, *Modeeria rotunda,* and one cnidarian was confirmed to species level, *Leuckartiara octona.* There were two specimens that were identified to species not recorded in Norway. This was a siphonophore, *Agalma clausi*, and a ctenophore *Beroe gracilis*. Hence, they were discussed as siphonophore indt. and *Beroe* spp. respectively (Artskart.artsdatabanken.no c, 2024). The last 11 sequences confirmed the morphological identifications.

A bioluminescent spectrum was obtained from 18 taxa, divided over four phyla: Annelida, Cnidaria, Ctenophora and Arthropoda (Table 3). The non bioluminescent taxa were *Modeeria rotunda*, *Aurelia aurita* and *Pleurobranchia pileus* (Claes *et al*., 2024).

Table 3: Overview of all the measurements performed on the identified taxa. The lowest certain taxon is described with the number of specimens found (n). This is followed by the integration time mode, giving an indication of the brightness for the flash. The percentage is based on the number of measurements performed with one method. Total success describes the total percentage of bioluminescent signals for each taxon and stimuli. The number of trials is the total number of measurements performed on each taxon and for each method. Note that it does not reflect the number of specimens since multiple stimuli methods were performed on some specimens. Fields with a - means that no measurements were performed with this method for the taxon.

TAXON (n)	INT. TIME	MECHANICAL	FRESHWATER	SOAP	LIGHT	TOTAL SUCCESS	NUMBER OF TRIALS
Aetideopsis armata (1)	2	100 %	-	-	-	100 %	1
Aurelia aurita (1)	1	0 %	-	-	-	0 %	1
Beroe spp. (14)	1	43 %	33 %	-	-	42 %	26
Bolinopsis infundibulum (11)	2	81 %	-	-	-	81 %	16
Bolinopsis spp. (4)	1	20 %	-	-	-	20 %	5
Clytia spp. (39)	1	45 %	0 %	-	-	41 %	49
Coelenterate indt. (2)	1	25 %	100 %	-	-	40 %	5
Ctenophore indt. (3)	2	100 %	-	-	-	100 %	3
Euphausiid indt. (27)	2	24 %	-	-	46 %	33 %	33
Euplokamis cf. *dunlapae (1)*	1	100 %	-	-	-	100 %	1
Hansarsia megalops (3)	1	100%	-	-	100%	100%	3
Hydrozoan indt. (3)	1	29 %	0 %	-	-	25 %	8
Leuckartiara octona (1)	1	0 %	0 %	-	-	0 %	3
Lobate indt. (8)	2	50 %	-	-	-	50 %	14
Mertensia ovum (2)	1	100 %	-	-	-	100 %	2
Metridia spp. (124)	2	73 %	32 %	0 %	-	62 %	149
Modeeria rotunda (1)	2	0 %	-	-	-	0 %	2
Pleurobrachia pileus (1)	1	0 %	0 %	-	-	0 %	2
Siphonophore indt. (3)	1	0 %	0 %	-	-	0 %	12
Thysanoessa raschii (1)	2	100 %	-	-	-	100 %	1
Tomopteris spp. (29)	2	21 %	0 %	19 %	0 %	18 %	57
TOTAL SUCCESS		52 %	22 %	14 %	47 %	46 %	
TOTAL NUMBER OF TRIALS		304	49	22	15	180	390

3.2 Stimulated bioluminescence

Of all the 390 measurements, there were 180 measurements (roughly 46%) that produced a recordable bioluminescent signal, independent of SNR. The most frequent (304 out of 390 measurements) and successful (52%) method used was mechanical stimuli (Table 3). Light stimulation also had a high success, but only for the euphausiids, including euphausiids indt. and *H. megalops.* Freshwater and soap (osmotic stress) were seldom used, and normally did not lead to any bioluminescent recordings, except in a few *Beroe* spp., Coelenterate indt., *Metridia* spp., and *Tomopteris* spp.

Ctenophore flashes were often quick and spread over the specimen's body, except for *Mertensia ovum*, who produced an external cloud of bioluminescence. No spectra was obtained from this specimen. The most successful method to elicit bioluminescence in this taxon was mechanical stimulation (Table 3). Freshwater was attempted for three *Beroe* spp. and one coelenterate indt., and only one *Beroe* spp. and the coelenterate indt. produced a bioluminescent signal. Few measurements were done with osmotic stress because the specimens would often dissolve or disintegrate. When considering taxa with more than three specimens, *B. infundibulum* (n=16, 81% success rate with mechanical stimuli), was the most easily stimulated in this study.

In the phylum cnidaria, only hydrozoans produced a viable signal, and only with mechanical stimulation. *Clytia* spp. were the second most frequent taxa found, and 41% of the 49 measurements gave recordable bioluminescent signals. The most successful method used was placing the specimens in small light tight tubes. Freshwater stimulation was attempted for a few individuals, but this did not lead to any bioluminescence.

Metridia spp. was the most numerous taxa, with 124 specimens and 149 measurements. Mechanical stimulation was the most frequent and successful method used for this taxon (Table 3). Most *Metridia* spp. flashed when lifting the Eppendorf tube out of the water and gently shaking it. Some required vigorous shaking, while others only needed to be lifted out of the water. Although they did not have the highest success percentage, it was methodologically the simplest taxon to stimulate, and the flashes normally lasted for several seconds, allowing multiple recordings per measurement. Osmotic stress was used multiple times, but with lower success rates (32% of freshwater measurements, and 0% of the soap measurements). Similarly, *A. armata* was measured in the same way as *Metridia* spp. and the specimen glowed with only brief mechanical stimulation.

H. megalops (n=3), had a 100% success rate for both mechanical and light stimuli (Table 3). For the euphausiids measured in Ny-Ålesund, including euphausiid indt. and *H. megalops*, a precise measurement with high proximity to the photophores was essential to obtain a signal. Light stimulation was performed by leaving them in an environment with dim light (Figure 10), and mechanically stimulating them if they did not glow when the light was turned off. Light without further stimuli only activate bioluminescence for a few specimens, but the subsequent mechanical stimuli was effective to activate bioluminescence. For the small euphausiids found in the Trondheimsfjord, including euphausiid indt. and *Thysanoessa raschii,* mechanical stimuli from gentle shaking of small tubes were a successful method.

Figure 10: Photo of two bioluminescent euphausiids that has been stimulated by light. Bioluminescence is produced from the ventral photophores (seen here as blue, shining spots), and the spectrometer fiber is placed close to the photophores to measure the spectral bioluminescence. Photo by Hedda Førde.

Subjectively, and when disregarding taxa where no bioluminescence was recorded, *Tomopteris* spp. was the most difficult taxon to stimulate and record. 29 specimens were found, but only 18% of the total 57 measurements were successful. Due to the low success rates, all methods were attempted. There was no success with freshwater or light stimuli. There were a few successful trials with dipping the specimen in soap water before mechanically stimulating it. The most successful method included lifting the specimen out of water and stroking the parapodia or mechanically stimulating them in small tubes, the same way as *Metridia* spp.

The measurements on size were not used (discussed in section 4.5.3). However, from qualitative observations, there was no correlation between size and signal obtained. The signal was instead dependent on if the measurement container was suitable for the specimen, and if the optic fiber was close or at the right angle to the specimen. Optimally, the distance is under one cm, and at a straight angle from the light producing organ.

3.3 Interspecific variation in spectral bioluminescence

Of the 180 recorded spectra, 139 were selected for further analysis because they had a clean signal (above SNR=2±0,1, Table 4). The bioluminescent taxa include two violet λ_{max}, 10 blue λ_{max}, four green λ_{max} and one yellow λ_{max}.

Ctenophore wavelength at peak intensity (λ_{max}) had a narrow range from 488,0 nm to 495,0±1,2, and a homogenous full band width at half intensity (FWHM) of 82,1–88,3 nm. *Euplokamis* cf*. dunlapae* had the shortest λ_{max} and FWHM of the ctenophores (λ_{max}=488,0 nm, FWHM=82,1 nm), followed by *Beroe* spp. (λ_{max}=494,2±0,9 nm, FWHM=88,0±1,1 nm) and *B. infundibulum* (λ_{max}=495,0±1,2 nm, FWHM=89,3±1,4 nm).

Clytia spp. is divided into three groups; two due to variation in the FWHM (see section 3.4) for further details) and one outlying value in FWHM, denoted as *Clytia* sp.* (λ_{max}=500,9 nm, FWHM=87,0 nm). The *Clytia* spp. found in the Trondheimsfjord had a λ_{max} of 502,4±1,5 nm and a FWHM of 51,0±0,9 nm, while specimens from Hopavågen had a λ_{max} of 504,2±0,5 nm and a FWHM of 46,2±0,9 nm.

Arthropod bioluminescence can be divided into two orders, the calanoids and euphausiids. From the calanoids, *Metridia* spp. (λ_{max}=489,5±0,3 nm, FWHM=81,6±0,3 nm) and *A. armata* (λ_{max}=420,9 nm, FWHM=69,4 nm) had a very different signal compared to each other, and A*. armata* had the shortest λ_{max} of all taxa examined. One outlier was found, marked with *Metridia* sp.* (λ_{max}=423,0 nm, FWHM=66,6 nm). In contrast, the three different euphausiid taxa had a similar spectrum: euphausiids indt. (λ_{max}=471,7±1,2 nm, FWHM=48,28±1,5 nm), *H. megalops* (λ_{max}=469,8 nm, FWHM=45,5 nm) and *T. raschii* (λ_{max}=472 nm, FWHM=52,3 nm).

Finally, one phylum of annelids was found, *Tomopteris* spp. They had a λ_{max} of 489,5±2,2 nm, and FWHM of 83,0±1,1 nm. There was one distinct outlier, *Tomopteris* sp.* in Table 4 (λ_{max}=567,4 nm, FWHM=36,4 nm). This was the specimen with the longest λ_{max} and narrowest FWHM in the study.

Regarding the SNR which gives an indication on the signal quality, there was a large variation between the taxa. *Metridia* spp. had the highest ratio (SNR=483,8), followed by *Clytia* sp*. (SNR=201,3) and *Tomopteris* sp.* (SNR=96,4). The rest of the taxa had a SNR below 40, hence over 12 times lower than the highest ratio.

Based on average λ_{max} and FWHM the measurements were divided into five main clusters of specimens (Figure 11). The largest and most diverse cluster has a λ_{max} in the blue-green color spectrum, around 475 nm to 510 nm, and a FWHM from 75 nm to 95 nm. It contains all the ctenophores, as well as one *Clytia* spp., and most *Metridia* spp. and *Tomopteris* spp. The second cluster is in the blue-violet color spectrum and only contains one order: the calanoids *Metridia* spp. and *A. armata.* The third cluster also only contains one order, the euphausiids. They are in the blue color spectra, and the figure shows that *T. raschii* has a slightly longer λ_{max} and wider FWHM than *H. megalops*. The two last clusters only contained one taxon. *Clytia spp*. was the sole taxon fully in the green spectral range (Figure 11), and the last *Tomopteris* spp. had a unique yellow bioluminescent signal.

Table 4: The average peak wavelength (λ_{max}), average full bandwidth and half intensity (FWHM), their respective standard error of mean (SEM), average signal to noise ratio (SNR) and number of specimens (n) included in the analysis. The table is divided into different phylum. The colors are divided as in Austin *et al*. (2021), only violet and blue are split up; violet: 350-450nm blue: 450-500nm, green: 500-565nm, yellow: 565-590nm is are * Shows the anomaly specimens, perhaps another species or just an outlier of its taxa. There was an intraspecific difference in *Clytia* spp. therefore they are divided by location, trd. were found in the Trondheimsfjord during August, and Hop. were found in Hopavågen during October.

TAXON	COLOR	λ_{max} (nm)	SEM λ_{max}	FWHM (nm)	SEM FWHM	SNR	n
Coelenterate							
Coelenterate indt.	Green	500,9	1,5	91,5	1,9	10,3	2
Ctenophora							
Beroe spp.	Blue	494,2	0,9	88,0	1,1	19,0	7
Bolinopsis infundibulum	Blue	495,0	1,2	88,3	1,4	16,9	13
Ctenophore indt.	Blue	489,0	1,5	86,1	2,1	37,4	2
Euplokamis cf. *dunlapae*	Blue	488,0	-	82,1	-	6,5	1
Lobate indt.	Blue	494,1	5,5	86,0	2,0	4,7	3
Cnidaria							
Clytia spp. [Trd.]	Green	502,4	1,5	51,0	0,9	28,0	2
Clytia spp.[Hop.]	Green	504,2	0,5	46,2	0,9	4,6	11
Clytia sp.*	Green	500,9	-	87,0	-	201,3	1
Arthropoda							
Aetideopsis armata	Violet	420,9	-	69,4	-	6,2	1
Euphausiid indt.	Blue	471,7	1,1	48,8	2,5	7,7	4
Hansarsia megalops	Blue	469,8	-	45,5	-	19,4	1
Metridia spp.	Blue	489,5	0,3	81,6	0,3	483,8	83
Metridia sp*.	Violet	423,0	-	66,6	-	4,0	1
Thysanoessa raschi	Blue	472,0	-	52,3	-	3,3	1
Annelida							
Tomopteris spp.	Blue	489,5	2,2	83,0	1,1	28,9	5
Tomopteris sp. *	Yellow	567,4	-	36,4	-	96,4	1

When considering the normalized spectral shapes, variation not detected by the λ_{max} and FWHM was observed (Figure 12). Only taxa taken to genus or species level are included to limit the generalization of higher taxa to the spectra of lower taxa. The ctenophores, *Beroe* spp., *B. infundibulum* and *E.* cf. *dunlapae*, all have a bell-shaped curve with few characteristic features. *Beroe* spp. and *B. infundibulum* have almost identical curves, with a slightly different λ_{max}. *E.* cf. *dunlapae* on the other hand, has a narrower curve compared to the two other ctenophores. *Clytia* spp. has a relatively narrow peak, followed by a shoulder at roughly 540 nm. *Metridia* spp. also has a relatively even bell-shaped curve, but the top has a smaller gradient from the λ_{max} to 465 nm. The other calanoid however, *A. armata*, has a relatively even bell-shaped curve at shorter wavelengths compared to *Metridia* spp. Both have a slight shoulder at 465 nm. The two euphausiids, *H. megalops* and *T. raschii* both have a narrow bell-shaped curve with a slight shoulder towards the longer wavelengths, but are still slightly skewed compared to each other, with different λ_{max}. Finally, *Tomopteris* spp. has a bell-shaped curve with a small gradient from the peak down to 465 nm, just like *Metridia* spp.

The Kruskal Wallis test confirmed that there are statistical significant differences is both λ_{max} (df=6, p-value=$2{,}7\text{x}10^{-12}$) and FWHM (df=6, p-value=$1{,}6\text{x}10^{-12}$), thus, post hoc comparisons (the Dunns test) were completed for both variables, and are presented in Table 5 for λ_{max} and Table 6 for FWHM comparisons. Of the in total 21 different comparisons, 15 were statistically significantly different from the compared parameter of the compared taxa (Table 5, Table 6).

The p-value statistics from the Dunns test indicate that the ctenophores, *Beroe* spp., *B. infundibulum* and Lobate indt. are indistinguishable from each other, when considering both λ_{max} and FWHM (p= 1,00 for all six comparisons). All three taxa are statistically different from the FWHM of *Clytia* spp. and euphausiid indt, but their λ_{max} is identical to *Clytia* spp. Although the ctenophores are statistically identical, only *Beroe* spp. and *B. infundibulum* have different λ_{max} to euphausiid indt.

The statistical results show that *Clytia* spp. has at least one significant different parameter to all taxa, while both parameters are unique compared to *Metridia* spp. The euphausiid indt. are also different to most taxa, but not significantly different to *Metridia* spp. and *Tomopteris* spp. *Metridia* spp. has relatively average parameters, because it is statistically alike both euphausiids indt. lobate indt. and *Tomopteris* spp. However, even more statistically identical to the rest of the taxa is *Tomopteris* spp. where the only significantly different parameter was the λ_{max} comparison to *Clytia* spp.

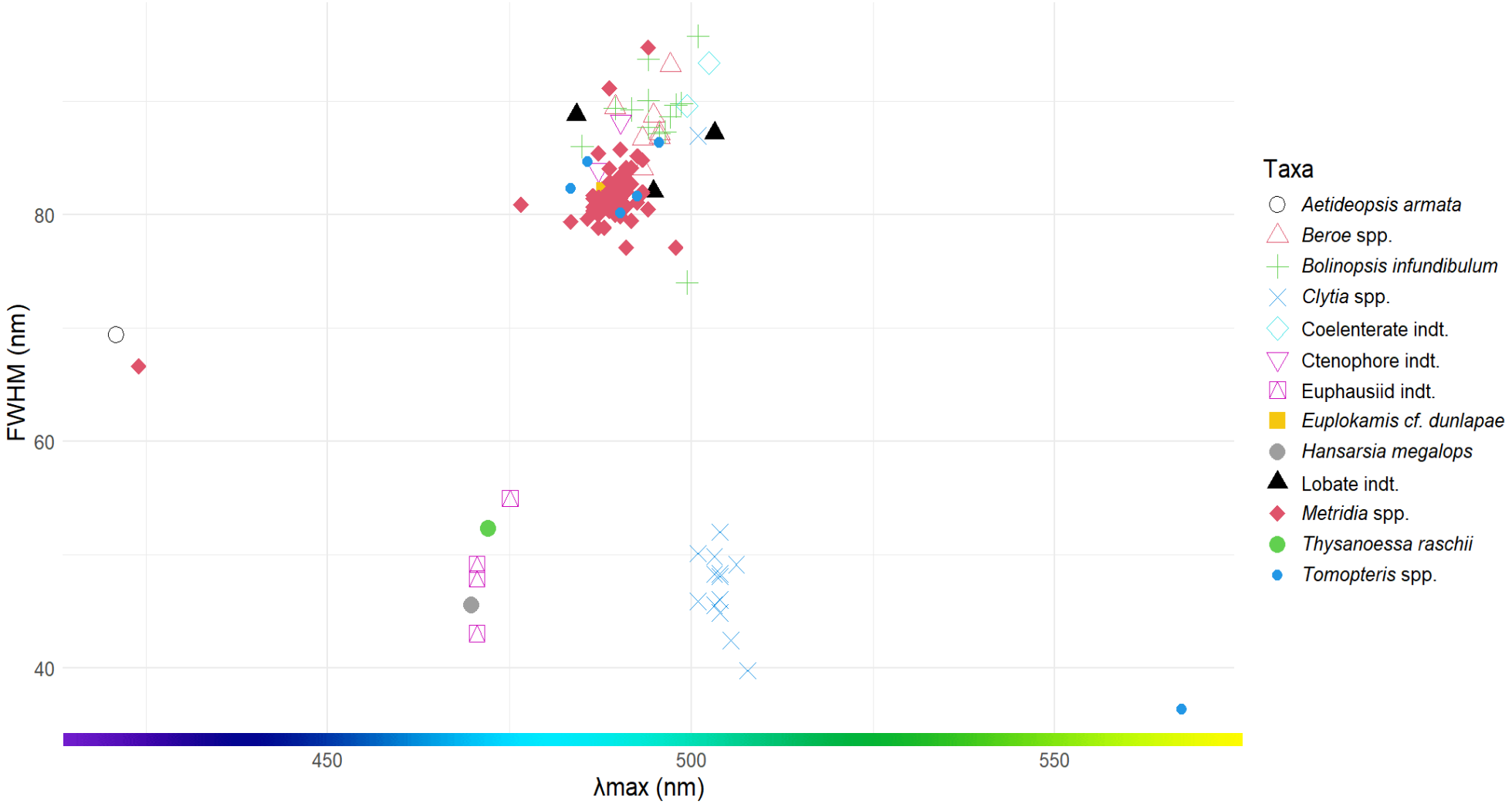

Figure 11: comparison of average peak wavelength (λ_{max}), average full bandwidth and half intensity (FWHM). Different shapes and colors are used to distinguish different taxa. The lowest taxa obtained for each specimen is presented, including the outliers. Spectral wavelength color bar (not to scale) is modified from ThoughtCo, Marina Li.

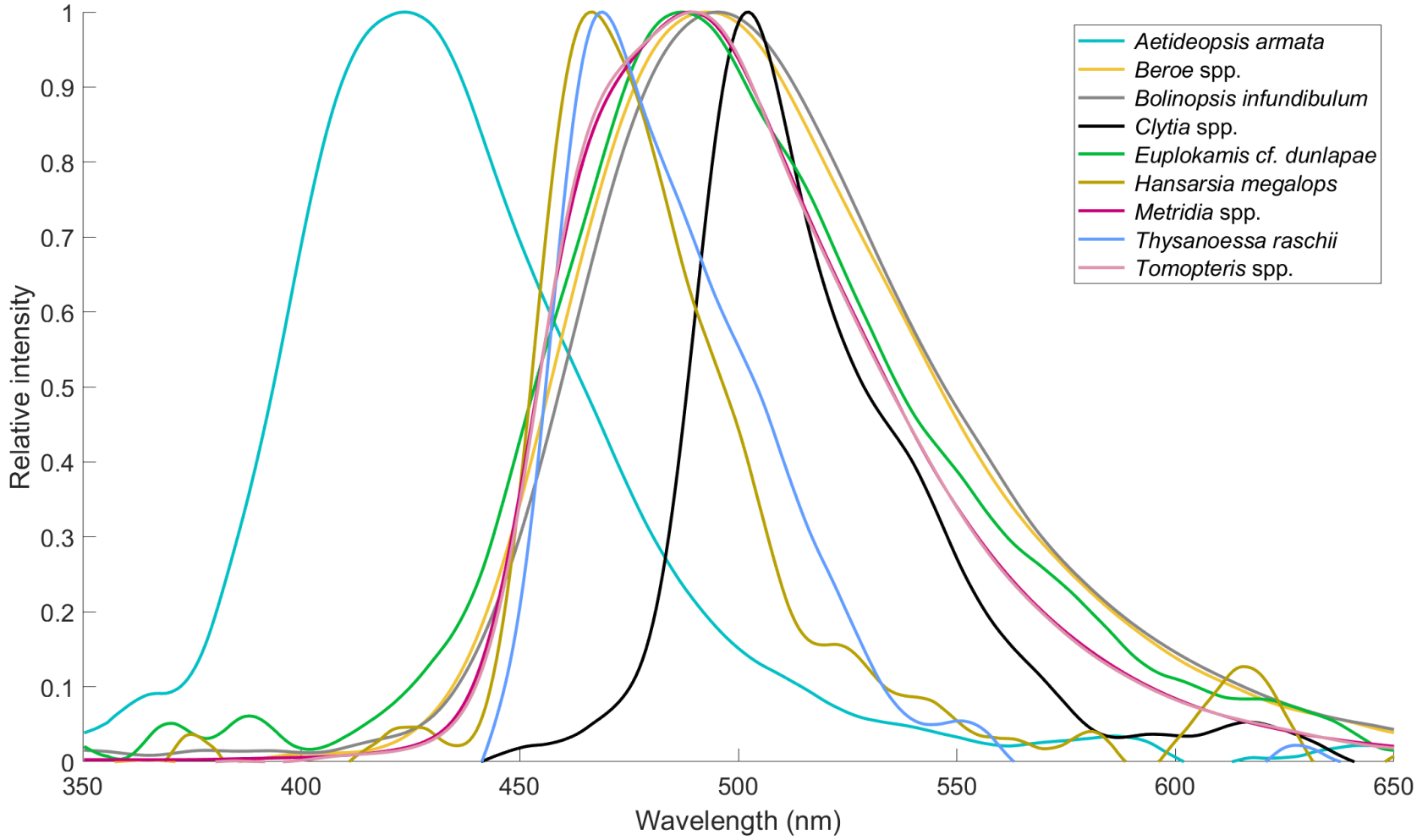

Figure 12: Normalized (to peak wavelength has a relative intensity of 1) spectra from the different taxa. One measurement from each taxon was chosen, based on the highest SNR. Only taxa with a SNR above 2 were chosen, and only the taxa that were identified to genus or species level. The peak around 610 nm is noise. The signal with the highest SNR was chosen as a representation of the respective taxa included in the figure.

Table 5: pairwise comparison of peak wavelength (λ_{max}) from the Dunn test. Only taxa with more than three specimens were included. A p-value below 0,05 means that the two comparisons are significantly different (color shaded boxes).

	Beroe spp.	*Bolinopsis infundibulum*	*Clytia* spp.	Euphausiid indt.	Lobate indt.	*Metridia* spp.	*Tomopteris* spp.
Beroe spp.		1,00	1,00	0,0024	1,00	0,074	1,00
Bolinopsis infundibulum	1,00		1,00	0,00056	1,00	0,0033	1,00
Clytia spp.	1,00	1,00		$3,6 \times 10^{-7}$	1,00	$4,4 \times 10^{-9}$	0,016
Euphausiid indt.	0,0024	0,00056	$3,6 \times 10^{-7}$		0,22	0,20	0,64
Lobate indt.	1,00	1,00	1,00	0,22		1,00	1,00
Metridia spp.	0,074	0,0033	$4,4 \times 10^{-9}$	0,20	1,00		1,00
Tomopteris spp.	1,00	1,00	0,016	0,64	1,00	1,00	

Table 6: pairwise comparison of full bandwidth at half of maximum intensity (FWHM) from the Dunn test. Only taxa with more than three specimens were included. A p-value below 0,05 means that the two comparisons are significantly different from each other (dark shaded boxes).

	Beroe spp.	*Bolinopsis infundibulum*	*Clytia* spp.	Euphausiid indt.	Lobate indt.	*Metridia* spp.	*Tomopteris* spp.
Beroe spp.		1,00	$4,7 \times 10^{-9}$	0,00023	1,00	0,012	1,00
Bolinopsis infundibulum	1,00		$6,0 \times 10^{-11}$	$5,1 \times 10^{-5}$	1,00	0,00031	1,00
Clytia spp.	$4,7 \times 10^{-7}$	$6,0 \times 10^{-11}$		1,00	0,0013	$2,5 \times 10^{-5}$	0,56
Euphausiid indt.	0,00023	$5,1 \times 10^{-5}$	1,00		0,021	0,13	0,11
Lobate indt.	1,00	1,00	0,0013	0,021		1,00	1,00
Metridia spp.	0,012	0,00031	$2,5 \times 10^{-5}$	0,13	1,00		1,00
Tomopteris spp.	1,00	1,00	0,56	0,11	1,00	1,00	

3.4 Intraspecific variation in spectral bioluminescence

From statistical comparisons of the intraspecific variation by month, location, and stimuli method, only *Clytia* spp. showed variation in FWHM. Three specimens were found in the Trondheimsfjord during August, and 11 in Hopavågen during October. However, because one of them had an outlying FWHM (FWHM=87,0 nm, see Table 4), only two specimens from August were included in the comparison. The average FWHM in the Trondheimsfjord was 51,0±0,9 nm, and 46,2±0,9 nm in Hopavågen. The comparison from the Dunns test between the two locations gave a significant p-value of p=0,03, indicating that there is a variation between the two locations during the two months.

The outlying *Clytia* sp.* (SBL126, λ_{max}=500,9, FWHM=87,0) had multiple deviating spectra during the measurement. This suggests variation in spectral bioluminescence, also within one specimen (Figure 13). Figure 13 shows the average spectra from specimen SBL126 (dashed line) as well as the other spectral recordings from the same measurement. There is an evident shoulder around 460 nm, but with varying intensities, and a shoulder at 540 nm with less variation in the intensity. There was no correlation between intensity or FWHM over time (i.e. early spectral recordings had a higher intense shoulder compared to later recordings).

Although there was no other intraspecific variation, there were a few outliers in the genera *Clytia* spp. (Figure 14A), *Tomopteris* spp. (Figure 14B) and *Metridia* spp. (Figure 14C). *Clytia* sp.* has a similar spectral curve to the rest of its taxa, but an additional shoulder around 465 nm. The outlier in *Tomopteris* spp. had a yellow spectrum and a vastly different spectral curve compared to the other *specimens*. The curve is characterized by a narrow FWHM and a clear shoulder close to 610 nm, almost becoming a secondary peak. The *Metridia* sp.* has a similar curve as *Metridia* spp., but similar λ_{max} and FWHM as *A*. *armata*.

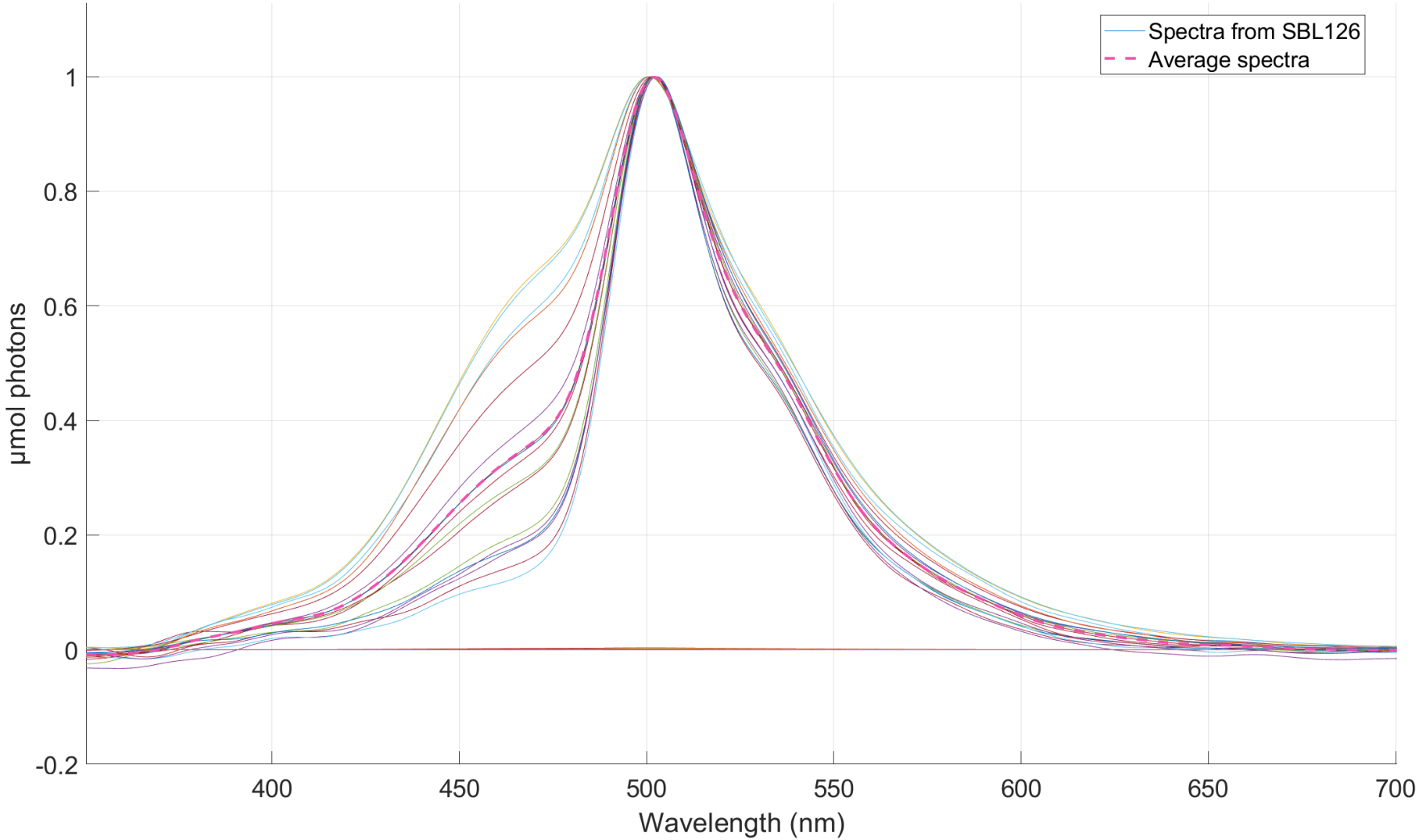

Figure 13: Normalized (at a peak emission of 1 µmol photon) spectral bioluminescence signal from *Clytia* sp., SBL126. All lines are different spectral recordings of the same specimen during the same measurement. The stippled pink line is the average spectra.

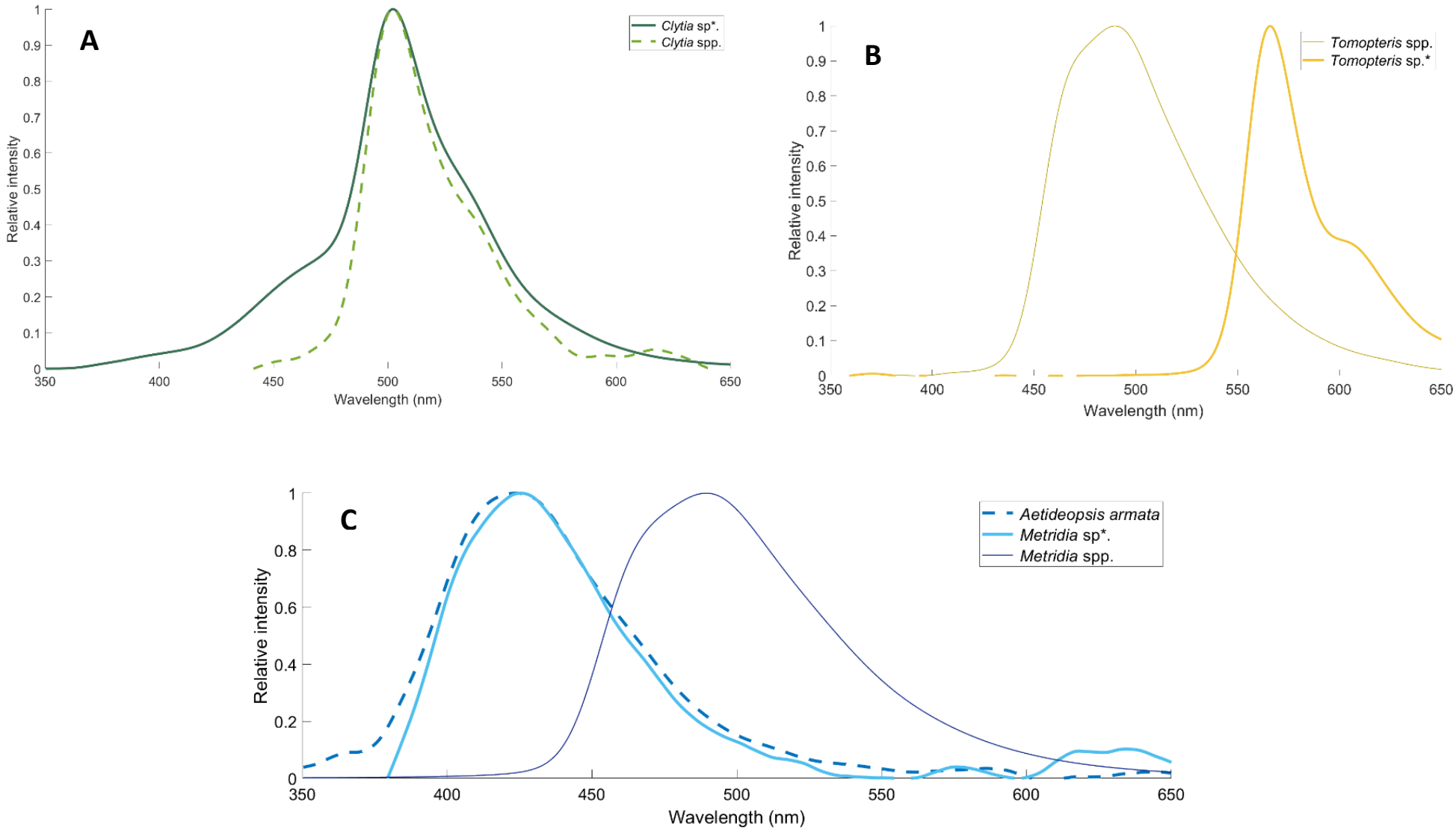

Figure 14: Normalized (to a relative intensity of 1) spectral bioluminescence from the outliers (genus maked with *), their plausible species spectra if available (stipled line), and example spectra from the taxa they have been morphologically identified to (thin line). The outliers found in this study are **A** *Clytia* sp*., **B** *Tomopteris* sp*. and **C** *Metridia* sp.*.

4 Discussion

4.1 Assessing biodiversity

Maintaining biodiversity and ecosystem functionality are the backbones of a healthy marine environment. This is one of the reasons that the rapid biodiversity loss has become one of the most urgent challenges we face today. It is therefore crucial to increase the understanding on our marine diversity and to learn how to protect it. However, with limited techniques for mapping and monitoring species diversity, distribution, and abundance, it becomes difficult to protect what is not known. Advancing methods and techniques to understand biodiversity is thus essential to take the next step into sustaining the ocean.

This study is the first (to our knowledge) to map zooplankton spectral bioluminescence in the Trondheimsfjord, Norway, over various locations and seasons, together with measurements from Kongsfjord, Svalbard, during polar night. It is shown that spectral bioluminescence could aid zooplankton monitoring, and it has the potential to be a valuable additional tool in the understanding of marine biodiversity. Today, due to multiple types of biases, zooplankton monitoring has some gaps in identifying certain taxa and understanding their abundance. For example, ctenophores are difficult to retrieve due to their fragility and are therefore completely missed and their abundances are often underestimated. Some taxa are difficult to identify further than phylum level due to morphological similarities. Adding spectral bioluminescence recordings to monitoring methods may help to solve some of these problems.

This will be discussed by first weighing the results from this study and other literature (further details in Appendix E), in context of how likely it is that the findings can represent the taxa.. For the taxa only taken to genus level, possible species are included based on what has previously been found in the same location. Thereafter, possible explanations will be presented for some of the variations in bioluminescence that has been observed, both in this study and between literature. This will be done considering both the ecological, physiological, and evolutionary perspectives, as well as the limitations and strengths of the methods used in this study. Then, all will be tied together, evaluating the extent to which spectral bioluminescence can be used to monitor zooplankton *in situ*, followed by a section for emerging questions.

4.2 Taxa specific comparisons

The phylum with the most bioluminescent taxa in this study were the ctenophores. They have a high abundance in the marine environment and close to 17% are luminescent (Claes *et al*., 2024). Hence, multiple studies have been conducted on their spectral bioluminescent signal (Haddock & Case, 1999; Herring, 1983; Latz *et al*., 1988; Widder *et al*., 1983). However, when comparing the genera and species found in this study, as well as between the latter studies, there are only a few spectral parameters that are either the same or within the standard error mentioned in the respective papers. For example, the *Bolinopsis infundibulum* measured by Haddock and Case (1999) had a seven nm shorter average λ_{max} than the specimens in this study. The SE and SEM does not overlap between the studies (Haddock and Case (1999): 488±4.2 SE, compared to this study: 495±1,5 SEM). In contrast, the FWHM was identical. The one specimen of *Euplokamis* cf. *dunlapae* in this study can only be compared to *Euplokamis* sp.

(Haddock & Case, 1999) due to lack of data on its spectral composition. They may be different species, and the parameters and errors between the studies do not overlap, but it is again seen that the FWHM measured is more similar than the compared λ_{max}.

Perhaps the most interesting phylum in this study was the cnidarians due to the variation between two groups of *Clytia* spp. A smaller percentage of the cnidarians are bioluminescent, only 1,29%, yet the phylum contains over a 100-fold more species than ctenophores. There are 85 hydrozoans that are assumed to be bioluminescent (assumed, meaning bioluminescence or light producing structures has been observed) (Claes *et al*., 2024), which is roughly 5% of all hydrozoans (MZGdb, 2024). Based on the molecular identification, two *Clytia* spp. from Hopavågen were identified as *Clytia hemisphaerica*, which according to Artsdatabanken, has been found in the study area (Artskart.artsdatabanken.no d, 2024). However, the molecular BLAST results showed the same likelihood between multiple results displaying *Clytia* spp. and a few *C. hemisphaerica*. Due to the high proportion of genus versus species levelresults, the taxon was only taken to genus level (Appendix C). The only other literature found on *Clytia* spectra was one specimen of *C. hemisphaerica*, and it had similar spectra to the specimens found in Hopavågen in this study (Haddock and Case (1999): λ_{max}=504 nm and FWHM=37 nm). Although most *Clytia* research has focused on *C. heisphaerica,* there are many other species in the genus, and new are constantly being discovered (Zhou *et al*., 2013).

The most numerous taxa in this study was *Metridia* spp. Based on visual inspection of the data, there seemed to be one clear outlier, hence, an interquartile range box and whiskers plot was made of all *Metridia* spp. λ_{max}. This showed that there were multiple outliers. The one extreme data point (*Metridia* sp.* in Table 4) was removed from the statistical analysis, but the rest were kept in the dataset because it may be natural variation and should therefore be kept in the dataset. Despite several outliers being included, this was the taxa with the lowest SEM of the λ_{max} and FWHM, indicating that the measurements are relatively precise. Due to low success rate in sequencing, the molecular identification did not provide any conclusions on which species the experimental specimens represented. Based on registered data in Artsdatabanken, most of the observations in study area have only been classified as *Metridia* spp., and a small percentage as *Metridia lucens* and *Metridia longa (Artskart.artsdatabanken.no a, 2024)*. The literature on their spectral bioluminescence found *M. lucens* to have a λ_{max} of 482 nm and FWHM of 85 nm, measured from crushed specimens (David & Conover, 1961). Similarly, Markova *et al*. (2004) found λ_{max}=480 nm for *M. longa.* These averages are seven to nine nm shorter than the *Metridia* spp. in this study.

Aetideopsis armata is an example of a specimen that was morphologically identified as a *Metridia* spp., it was considered an outlier due to its short λ_{max}, but from molecular identification, it was confirmed to *A. armata*. This result shows the potential of bioluminescence *in situ* taxa identification when some easily confused taxa can have distinct bioluminescent signals. On the other hand, there is still the unidentified outlier, also with a violet signal, and the spectral shape is slightly different to *A. armata* (Figure 14C). Although no studies were found on the spectral bioluminescence on *A. armata*, the only other arthropod that was found to have a similar λ_{max} was the amphipod (order) *Scina* (genus) where the λ_{max} ranged from 435 to 444, and FWHM of 64-89 (Herring, 1983; Latz *et al*., 1988; Widder *et al*., 1983). According to Artsdatabanken, the bioluminescent *Scina borealis* is present in the

Trondheimsfjord, and may consequently be misidentified if this were to be done *in situ* (Artskart.artsdatabanken.no e, 2024; Herring, 1967).

Also due to the molecular identification, the euphausiid indt. specimens in the measurements were reidentified to be *Hansarsia megalops* and *Thysanoessa raschii*. Both *H. megalops* and *T. raschii* had similar spectral parameters to Herring (1983) and Boden and Kampa (1959) respectively, but the λ_{max} of *H. megalops* was almost identical to the spectra in Herring (1983). Since none of the species were included in the pairwise comparison because too few specimens were species identified, no further conclusions can be made if the two taxa are distinguishable (Table 5, Table 6). However, it was in general seen that the λ_{max} of the euphausiids were similar, but there was large variation in FWHM (Figure 11).

The *Tomopteris* spp. on the other hand, was not identified further than genus due to lack of knowledge on certain morphological features and poor results from the molecular identification. Most of the tomopterids found had a blue bioluminescent signal. Gouveneaux *et al.* (2017) showed that most species have a yellow bioluminescent signal, except one, *T. planktonis*, with a blue spectrum, which is found in Western Norway. However, compared to the present studies' blue tomopterids, they measured the λ_{max} to be 39,5nm shorter, and the FWHM to be 60nm smaller. When considering appearances in the Trondheimsfjord, the specimens found have rarely been classified further than genus level, with exceptions of the yellow bioluminescent *T. helgolandica (Artskart.artsdatabanken.no b, 2024; Gouveneaux et al., 2017; Gouveneaux & Mallefet, 2013)*. Although one of the specimens in this study had a yellow spectrum (Figure 14B) and similar morphology as T. *helgolandica*, the uncertainty was still too high for species level taxonomy. The yellow spectral curve obtained is also similar to measurements by Francis *et al.* (2014) in Monterey Bay, USA, where the specimens also were not further identified than to *Tomopteris* spp.

4.3 Spectral bioluminescence in the light of literature

To meaningfully compare spectral composition between studies, a range of variables need to be accounted for. Multiple hypotheses could be discussed based on the different instruments used from both the 20th and 21st century, the stimulation methods, the locations and depths specimens were found at, as well as criticism to how the species were taxonomically identified, the lack of information on stimuli success, as well as number of specimens measured.

The methods used to measure bioluminescence may be a central reason for the spectral variation between, and within, different studies. For example, Gouvenaux *et al*. (2017) measured *T. helgolandica* in two ways; three specimens from a digital picture and one live measurement. The λ_{max} recorded was the same for both, but the FWHM varied from 130 nm in the digital picture, and 51 nm from a live recording.

Another aspect of measurement method is the instrumentation used and may ultimately be one of the main limitations in obtaining a reliable spectra. In the present study, the spectrometer has a spectral resolution 0,77nm, giving one or two measurements per nm. However, in some studies, the spectral resolution was up to 20 nm. For example, Widder *et al*. (1983) measured a few *Beroe* species, with an optical multichannel analyzer (OMA). Depending on the size of the slit ("opening") at the end of the

optic fiber, the measurement resolution deviated from 2 nm to 9 nm, or up to 20 nm if an alternate lens was used, or no collection optics (Widder *et al*., 1983). Three different studies found the ctenophore *Beroe cucumis*, but all had different λ_{max} varying by 10 nm from the shortest to longest average, despite all referring to OMA device used in Widder *et al*. (1983) (Haddock & Case, 1999; Latz *et al*., 1988; Widder *et al*., 1983). Widder *et al*. (1983) had a resolution of 20 nm for its *B. cucmis* measurement, Latz *et al*. (1988) had a resolution of 9 or 20 nm (not specified for *B. cucumis*) and Haddock and Case (1999) did not mention the slit specifics.

Despite several studies utilizing OMA and consistently referring to Widder *et al*. (1983) for further details, there are still considerable variation between the studies. While biological factors, such as specimens collected from different depths and locations, may contribute to this variation, it highlights the need for a consistent and taxa specific method protocol. This would allow comparison of literature and methods. Only then can biological variation be considered as the primary cause of spectral variation.

4.4 Spectral bioluminescence in the light of biology and ecology

In addition to method being a likely cause of the variation between literature, it is known that the spectral bioluminescence can vary due to several biological and ecological factors.

Intraspecific variation due to phenotypic plasticity may play a role in bioluminescence. This could mean that there is an effect of different functional traits at different depths, locations or seasons. For example, the *B. infundibulum* (λ_{max}=488nm±4.2 SE) found by Haddock and Case (1999) were at 100 m depth, where the light climate is most likely blue-green. The *B. infundibulum* found in this study (λ_{max}=495 nm±1,2 SE) was at the surface of the bay, Hopavågen, where the light climate was green. Although both *B. infundibulum* spectra are in the blue-green color spectrum, the specimens found in Hopavågen had a greener spectrum compared to the deep dwelling specimens which have a bluer spectrum, correlating to the spectrum of light of their environment.

Similarly, if we assume the different Clytia spp. found are the same species, the intraspecific variation between Hopavågen and the Trondheimsfjord may also be due to phenotypic plasticity. *Clytia* spp. had an interesting variation in FWHM, with multiple deviating widths within one specimen, as seen in Figure 13. This may be due to the green fluorescent proteins. Similar results have been seen in laboratory experiments of the species *Clytia gregaria* (Markova *et al*., 2010). Markova *et al*. (2010) found that the photoprotein Clytin in *C. gregaria* had a λ_{max} of 475 nm. However, when a clone of the species GFP was measured, there was a λ_{max} close to 500 nm and a blue and green shoulder by 468 nm and 540 nm, respectively. In the study, the blue 468 nm shoulder decreased in intensity when there was a higher concentration of GFP, meaning the spectra would appear greener when there was more GFP. Two of the Hopavågen *Clytia* spp., including the one marked as an outlier, had a shoulder at roughly 465 nm and 540 nm, while the rest only had a shoulder at 540 nm (Figure 14A). These results could imply that a greener spectrum, i.e activated GFP, is more apparent in Hopavågen during October. The light climate in Hopavågen was greener than the Trondheimsfjord, so if all the *Cytia* spp. are the same species, this could be a result of phenotypic plasticity.

If not phenotypic plasticity, the intraspecific variation may be due to genetic variance or speciation. It has been suggested that speciation in the pelagic zone is driven by habitat selection, rather than

physical barriers (Peijnenburg & Goetze, 2013). The compared spectral composition has been from species in different parts of the world, meaning there could be genetic variation in bioluminescent signals due to varying light climates or habitats. Since bioluminescence has evolved individually over 40 times, it is assumed that its evolution is relatively easy. (Haddock *et al*., 2010; Widder, 2010). Furthermore, since there is a close correlation between vision, light climate and bioluminescence (Frank & Widder, 1999), there are grounds to believe that there is some coevolution between the different factors, ultimately altering species bioluminescence. Perspectives on evolution are important because it is uncertain exactly how zooplankton adapt to their environment, or to a changing environment. Several studies show that zooplankton respond to warmer climates by changing their distribution or phenology, while evolutionary adaptations to their habitats have often been overlooked, but may also be a significant perspective to endulge (Dam, 2013; Helaouet & Beaugrand, 2009; Peijnenburg & Goetze, 2013).

In addition, knowing that bioluminescence can be dependent on diet (Haddock *et al*., 2010), it could mean that food sources are different between the locations. Euphausiids have most likely gotten their bioluminescence from consuming dinoflagellates, and when comparing dinoflagellate species-specific spectra, there is also variation between studies (Seliger *et al*., 1969; Widder *et al*., 1983). Seliger *et al*. (1969) studied the spectra in cultured dinoflagellates, while Widder *et al*. (1983) measured bioluminescence in the same species but found them off the coast of California. The spectra varied by eight to five nm between the two studies. Given that the variation is natural (not methodological or instrumentational error), this shows that there is a difference in spectra within species due to environment since one is cultured and the latter is first-generation wild specimens. If the bioluminescence varies by diet, perhaps this could affect the consumers' bioluminescence as well.

During the measurements of this study, not all specimens would produce a bioluminescent signal. The circadian rhythm, seasonality or life stage can be reasons for why some specimens produced a dimmer flash than others or did not glow at all after stimuli. Several studies have shown that dinoflagellate bioluminescent potential is dependent on their circadian rhythm, where the flashes are brighter during the dark phase (night) compared to the light phase (day) (Anderson *et al*., 1988; Christianson & Sweeney, 1972; Hastings, 2013; Latz & Lee, 1995). Accordingly, Krohn-Pettersen (2023) performed multiple *in situ* experiments on bioluminescence flash kinetics in Hopavågen, and found that most specimens had a significantly brighter flash during the night, than during the day. One plausible reason is presented by Berge *et al*. (2012), where they find evidence for bioluminescence being inhibited when the ambient light is stronger than the bioluminescence produced from the specimen itself. Hastings (2013) however, suggests that the variation between night and day, at least in dinoflagellates, is most likely because flashing is an unnecessary feature during the light phase. However, during the polar night in Kongsfjord when no sunlight was apparent, one study showed that there was no evidence for a change in bioluminescent potential (Berge *et al*., 2012). Hence, the spectral measurements performed in this study during the polar night may not be affected by a change in bioluminescent potential, but ones performed by the Trondheimsfjord may be affected, as shown by Krohn-Pettersen (2023).

It is probable that the observed variation in spectral bioluminescence between and within taxa is due to inconsistencies in literature and biological variation. However, the methodology in the present study also needs to be considered and may have implications for the results obtained.

4.5 Methodology

4.5.1 Species composition and taxonomical classification

The species composition in this study was only holoplankton. The fieldwork was coordinated with campaigns of other projects, so the sampling was opportunistic. Ideally, more locations and time points would have been used to collect specimens. In addition, when the specimens were sorted and identified to the lowest classification possible with minimum disturbance, only the individuals belonging to known bioluminescent taxa were chosen for spectral measurements. This was done because mechanical stimulation does not trigger bioluminescence in all zooplankton, thus, it would be time consuming to stimulate multiple specimens of unknown taxa. Hence, there was a focus on obtaining quality data on fewer taxa, rather than testing as many taxa as possible.

Regardless, several of the bioluminescent species in Kongsfjord and Trondheimsfjord were found in this study, but there are also many missing (Claes *et al*., 2024; Hop *et al*., 2019). For example, the invasive *Mnemiopsis leidyi*, which is a morphologically similar ctenophore to *B. infundibulum.* This species is classified on the Norwegian Foreign Species List 2023 to pose a "very high risk" to native species and ecosystems (Falkenhaug *et al*., 2023). This example shows how important it is to map as many bioluminescent species as possible, to avoid misconceptions and false interpretation of spectra. Thus, if *in situ* spectral measurements are to become a tool, the reference database needs to include a broad region to detect the migrators.

When identifying the species, there were a few challenges. The morphological identification was often limited because the live specimens needed to be in optimal conditions for stimuli, so they were only briefly identified before the measurement. After the measurements, some were damaged, increasing the difficulty of morphological identification. Hence multiple specimens were classified with "indt.". In addition, there was a limited amount of EtOH in Ny-Ålesund, so no Kongsfjord specimens of *Beroe* spp. and other coelenterates were preserved for molecular identification.

The molecular identification answered many questions about what species were found, like reclassifying two outliers of *Metridia* spp. However, due to manufacturers' mistake with the kits used to extract the DNA, fewer results were obtained than originally planned. Consequentially, not all specimens with a deviating or average bioluminescent signal could be identified to species level. Furthermore, from the BLAST results, *A. armata* was classified as *Chiridius armatus*, which is an outdated name (Walter & Boxshall, 2024). Hence, *A. armata* is used instead in this paper, but it is described as *C. armatus* in (Appendix C). Lastly, due to many of the spectral bioluminescence studies being relatively old, the *H. megalops* identified from BLAST, has been described with its previous name, *Nematoscelis megalops* in the reference literature (Herring, 1983; Siegel & De Grave, 2024).

In general, there is a high uncertainty regarding species level identification. For example, most of the literature presented in section 4.2, failed to describe how the specimens were identified, and if molecular methods were used. The fine balance between the pre-measurement morphological

identification and the risk of accidental stimuli, versus ability to morphologically identify specimens after measurements, is also a likely issue in the compared papers. Haddock and Case (1999) and Francis *et al*. (2014) were exceptions, and both mentioned how the ctenophores and *Tomopteris* spp., respectively, are complex species to identify with deviating information on morphology and genetics. The uncertainties pose a large problem because they limit our understanding of species biology and ecology. This knowledge is the foundation to understand how zooplankton disperse with changing climates and understanding invasive species management (Johansson *et al*., 2018).

4.5.2 Data analysis

Even though the result from this study often deviates from literature, the SD and SEM between the studies are similar, and commonly lower in the present study. The error might be lower in this paper because the instrumentation was relatively precise (resolution of 0,77nm), in contrast to studies using an OMA with up to 20nm resolution. A low resolution can therefore lead to an unprecise λ_{max} and FWHM.

Another difference between studies can be how the spectral curves were smoothed. Most studies did not mention how this was done. When different smoothing methods were tested in this thesis to find the best calculation, the λ_{max} changed slightly, up to 1 nm. The current smoothing method described in the methods (section 2.5.1) was chosen by using a calculation that limited changes in unique features of spectral curves.

When using the SNR to choose spectra for final analysis, no studies mentioned how this was calculated, or if they set a minimum SNR. Rather, signals with the best SNR was chosen for analysis (Haddock & Case, 1999) or curves were smoothed multiple times depending on the SNR (Widder *et al*., 1983). In this study, all spectra were treated equally, and the minimum SNR was set to 2, with one exception of one spectrum that had SNR=1,9. One advantage of the methods used in this study is that most noisy data is efficiently eliminated. However, a disadvantage is the sample size decreases, and dim flashes, which can still have a correct λ_{max} and FWHM, are eliminated.

4.5.3 Measuring bioluminescence

One of the biggest challenges with spectral bioluminescence is the measurement, because the flashes are often dim and sudden. Only 46% of all measurements performed gave a bioluminescent recording, including the number of times where a flash was seen, but not recorded. The sensor needs to be close; preferably less than one cm away, and at a good angle to the specimen, a frustration from several spectral bioluminescent studies (Widder 1983). To solve this, an optimal chamber for measuring bioluminescence is essential, as well as high quality instruments.

The most important properties of a container were volume, fiber proximity to the specimen defined by lid or opening size and the containers' reflective ability. This is central because the specimen needs to have some free movement, but not too much so that the specimen is difficult to detect for mechanical stimuli and recording. Keep in mind that recordings are done in a completely dark room, so it is important to know where the specimen is. Reflection is important because several specimens emit a dim flash, and the reflective ability of the container increases the likelihood of recording a signal.

During this study, measurements on size were made, but this data was not used due to challenges in obtaining precise measurements. Most specimens were measured after experiments to minimize handling, but if some were damaged, the original size was unknown. In addition, coelenterates quickly change their observed shape and size. However, how dense the emitted flash was on the specimens' body seemed to be important. For example, smaller ctenophores were often easier to obtain a signal from because the flash was more concentrated in the measuring container, allowing the fiber to detect the signal more easily. This is also a likely reason why *Metridia* spp. was the easiest to measure. An optimal chamber was found which allowed high proximity to the specimens, as well as a narrow opening to limit ambient light and noise. Consequentially, they had the highest signal to noise ratio.

From trial and error, it was found that even though a method could work theoretically, or in other studies, the practicality could be different. For example, the initial stimulation method for ctenophores was based on the theory behind a two-compartment integration sphere, where it was expected that the signal would be reflected from the specimen and to the optic fiber (Bowlby *et al*., 1991). Thus, small containers were 3D printed, with a sphere inside. This method was unsuccessful, perhaps because the space between the sensor and the specimen was too large and the flash was too dim. The same was observed for mechanical stimulation, where the effect of "mechanical" stimulation varies by the specific mechanical method. For example, the tomopterids in this study were mainly stimulated when the specimen was lifted out of the water and the parapodia was touched with something completely dry. Similarly, osmotic stress was used because other studies have claimed that it is effective (Chen *et al*., 2007; Gouveneaux *et al*., 2017; Heerklotz & Seelig, 2000), but this was not the case here. One study on the dinoflagellate *Gonyaulax* showed that sudden motion or centrifuging was not enough to mechanically stimulate the specimen, but rather shock waves. This deformed the cell membrane, concluding that it is how the stimuli physiologically affects the specimen that is important in stimulating bioluminescence (Latz & Lee, 1995). Thus, the full details of the method seem important, as well as how it is supposed to affect the specimen, further stressing the need for a taxa specific protocol of how to stimulate bioluminescence.

Nevertheless, one danger with alternating between stimulation methods is its effect on the bioluminescent signal. Knowing that some species have optical filtering in their photophores (Wilson & Hastings, 2013), different chemicals or stimuli may alter the cell properties, leading to different signals being recorded when the cell is intact versus damaged. Thus, if the specimens were left longer in the osmotic stress treatments, perhaps a flash would have been recorded due to puncturing cells, but it may not be relevant to the specimens' natural spectral bioluminescence. For instrance, in the study by David and Conover (1961) examining *Metridia lucens*, they crushed their specimens before measuring them, and in the studies done on *Tomopteris* spp., specimens were anesthetized (Gouveneaux *et al*., 2017). It is unknown how these chemicals and cell destructions affect the bioluminescence; therefore, their results may not provide relevant information to *in situ* measurements.

Aluminum foil was often used to increase reflection and block out external light sources. The danger of using reflective material is that it can cause emitted light to reflect through the specimen, or it can absorb light. With potential optical filtering, the light can be "double reflected" influencing the bioluminescent signal recorded. Therefore, measurements with and without a reflecting material may

vary. Unfortunately, the reflectance spectrum of the aluminum foil used in the measurements was not determined.

Finally, although recording the flash is challenging, some specimens may have lost their bioluminescent potential. In addition to innate biological reasons described in section 4.4, it could be due to random individual factors or welfare. Changes in temperature or light exposure can reduce fitness or bioluminescent potential. None of the studies that were used as a reference included information on how they sorted their specimens, i.e. in white or red light. It is therefore uncertain to what extent this affects bioluminescence.

This tied together, although there are spectral differences between taxa, obtaining viable signals was challenging, raising multiple questions to the practicality of how this will be done *in situ*.

4.6 Taxa recognition based on *in situ* measurements of spectral bioluminescence

Based on this study, the results show that spectral bioluminescence has potential for *in situ* taxa recognition. The predictable measurements in this study, are that euphausiids, *Clytia* spp. and *A. armata* can be distinguished from all taxa when judging from a λ_{max} and FWHM plot (Figure 11). Although there are some variations within the ctenophores, no clear pattern was detected (Figure 11, Table 5, Table 6). *Metridia* spp. was statistically distinguishable from *B. infundibulum*, and *Clytia* spp. when considering both spectral parameters (Table 5, Table 6). However, by looking at its spectral curve, *Metridia* spp., together with *Tomopteris* spp., has a unique, flatter gradient near the peak, compared to the typical bell-peaks, distinguishing them from other taxa.

The variation within, and between species is important because it contributes to a holistic understanding of all bioluminescent species. If spectral bioluminescence is to become a tool for identifying taxa, the measurements need to be predictable. Although some are, the bioluminescent community is not fully understood in the Kongsfjord and Trondheimsfjord. If blind tests were to be done on spectral bioluminescence, it would be, from a statistical perspective, uncertain if a ctenophore, *Metridia* spp. or *Tomopteris* spp. was observed, but more certain if an euphausiid or *Clytia* spp. was present. If suddenly cryptic or new species were measured, it would either be falsely classified or become an outlier. In addition, if multiple species were measured simultaneously, this would also pose a new challenge and lead to unclassified peaks.

Combining flash kinetics in some way with spectral composition could be promising. This makes sense both ecologically and evolutionally because of the small adaptations that can occur within the same optic environments, as seen for the coexisting *T. helgolandica* and *T. planktonis,* with a yellow and blue spectra, respectively (Gouveneaux *et al*., 2017). Combing the traits allows for more species to be examined, since Krohn-Pettersen (2023) and Johnsen *et al*. (2014) were able to identify some specimens that were not distinguishable here, and vice versa.

In situ measurements of bioluminescence have previously been attempted by using a mesh that moves in the water. Letendre *et al*. (2024) summarized the method. As the specimens were touched, they flashed, and the bioluminescence was recorded with a low-light camera and an infrared camera. The low-light camera would capture the flash, while the infrared camera would photograph the specimen, without bioluminescence being visible. Another tested approach was passive detection in the water

masses (Letendre *et al*., 2024). However, one limit of these two methods is that it is uncertain how the already existing light climate in the water column can impact the light recorded.

For now, the main challenges to be solved are how to stimulate natural bioluminescence, and how to measure it. The distance between the optical fiber and the specimen should preferably not be more than a few mm, and up to one cm, depending on the brightness of the flash. Therefore, for *in situ* measurements to be feasible, the recordings need to be performed in a dark environment, the spectral recording technology needs to have a high sensitivity, perhaps with different recording nodes that have different integration times. Lastly, there should be a mechanism that creates turbulence, to stimulate bioluminescence. As Letendre *et al*. (2024) mentioned, adding an infrared camera would allow further ground truthing.

If *in situ* measurements are not possible, laboratory experiments may still be sufficient for taxa recognition. It could be a useful additional tool to distinguish morphologically similar species.

4.7 Future perspectives

After having done hundreds of measurements on bioluminescent zooplankton, and reading through multiple research papers, a few questions for future studies have emerged.

Bioluminescence has evolved possibly over 40 times (Haddock *et al*., 2010), stressing that the trait can be essential for multiple species. However, it is unknown how a change in light climate can affect bioluminescence. The light climate can change in the oceans by increased artificial light, darkening of coastal waters, and decreasing sea ice allowing light to penetrate the sea during longer times of the year. Bioluminescence affects many biological processes, such as intra- and interspecific communication, for example counterillumination for camouflage. Other than the bioluminescent species themselves, the trait may be important for visual predators likes seabirds and fish to detect their pray (Johnsen *et al*., 2014). If the light climate changes, the function of bioluminescence could be altered, either because the color perception changes due to coastal darkening, that their bioluminescence is not visible with high presence of artificial light, or that zooplankton need to disperse to deeper water. One ripple effect of this is that the species that rely on zooplankton in the surface might struggle to find pray, creating a phenotypic mismatch. Research focusing on coastal darkening and changing light climate together with bioluminescence could give insight into the consequences of this. One way this could be done is by evaluating bioluminescence phenotypic plasticity. Phenotypic plasticity could explain some of the bioluminescence variation in this study, as well as giving information on how bioluminescent zooplankton adapt to their environment. Furthermore, if a passive spectral recording device was deployed for long term monitoring, and if a change is seen in the spectral composition or flash kinetics, this could regardless indicate a change in light climate or species composition without understanding what species are there. Perhaps specimens need to change their spectra or flash in order to be perceived by their own or other taxa.

Ultimately, if spectral bioluminescence is to become an *in situ* taxa recognition tool, further research is needed on all bioluminescent species found in a large surrounding area. An additional protocol for laboratory measurements is needed, to ensure a standardization between different research. Finally, the stimulation method needs to be done in a way so that can be implemented *in situ*.

5 Conclusion

To conclude, there is a high potential for *in situ* taxa recognition by using spectral bioluminescence. There is a statistically significant difference when considering λ_{max} and FWHM to distinguish ctenophores, euphausiids and *Clytia* spp. Furthermore, statistically similar species may have other features that give them a unique bioluminescent spectral shape, like *Tomopteris* spp. and *Metridia* spp. compared to ctenophores. There is little to no intraspecific variation between the methods used to stimulate bioluminescence, and between the sampling locations and months. However, the bioluminescent potential may be affected by these three factors. The FWHM of *Clytia* spp. showed a statistically significant difference between August in the Trondheimsfjord and October in Hopavågen. This may be due to a different expression of their green fluorescent proteins, or perhaps they are different species.

The results obtained were not always consistent with spectral measurements in other literature. Normally, the variation was around five to 10 nm in the λ_{max}, while the FWHM was more inconsistent. There was also variation between other studies that measured the same species, using the same measuring device. These variations between literature may be due to different stimuli and recording methods, or that there is biological variation, like genetic variation or phenotypic plasticity, over larger areas than considered in this study. Thus, to eliminate researcher biases, there is a need for taxa specific protocol on how to stimulate and measure bioluminescence. With a standardized method, it is more likely that true biological variation in spectral bioluminescence can be identified. However, the protocol can not only be designed to stimulate and measure bioluminescence in laboratory conditions, but it also needs to reflect a natural *in situ* bioluminescent signal.

The next steps into performing *in situ* measurements is firstly, to map all the bioluminescence in the relevant area, reducing the chance of falsely identifying species or obtaining outliers, like the yellow bioluminescent *Tomopteris* sp. or the violet *Metridia* sp. in this study. Secondly, the "proximity challenge" needs to be solved. In this study, only 46% of all the measurements led to a bioluminescent recording. This was either due to unsuccessful stimuli, no signal being recorded despite the specimen glowing, or a poor measurement resolution (i.e. too low signal to noise ratio). This low percentage stresses the need for reliable stimuli method.

Solving these challenges and building on the results obtained in this study, can lead to a bright future for the bioluminescent zooplankton. The current state of knowledge helps understand this understudied group of organisms by allowing spectral bioluminescence to be an emerging taxa recognition tool, facilitating the preservation of the ocean's biodiversity.

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7 Appendix

Appendix A: BLAST results from molecular identification. BL recorded is if bioluminescence was recorded during measurements, regardless of SNR. The author that described the species is mentioned the first time each specie is stated in the BLAST match. The first two BLAST results are always shown, and if the Query and Match percentage are the same for another more likely taxa, based on location, it is also added with its result number. Same region indicates if the BLAST match has been found in the same region as the morphological ID taxa.

MARKER	MORPHOLOGICAL ID.	SAMPLE ID	BL RECORDED	RESULT NUMBER	BLAST MATCH	QUERY	MATCH	AC NUMBER	SAME REGION
18S	*Bolinopsis infundibulum*	SBL042	Yes	#1	*Bolinopsis microptera (Agassiz, 1865)*	100 %	99,25 %	XR_010160386.1	No
18S	*Bolinopsis infundibulum*	SBL042	Yes	#2	*Kiyohimea usagi (Matsumoto & Robison, 1992)*	100 %	99,25 %	MW647045.1	No
18S	*Bolinopsis infundibulum*	SBL042	Yes	#8	*Bolinopsis infundibulum (O.F. Muller, 1776)*	100 %	99,25 %	MW647030.1	Yes
18S	*Beroe* sp.	SBL044	No	#1	*Beroe gracilis (Kunne, 1939)*	100 %	99,71 %	MF599317.1	No
18S	*Beroe* sp.	SBL044	No	#2	*Beroe forskalii (Milne Edwards, 1841)*	100 %	99,71 %	AF293697.1	No
18S	Ctenophore	SBL140	No	#1	*Modeeria rotunda (Quoy & Gaimard, 1827)*	98 %	99,90 %	FJ550540.1	Yes
18S	Ctenophore	SBL140	No	#2	*Ptychogena crocea (Kramp & Damas, 1925)*	100 %	96,26 %	KY363983.1	Yes
18S	Lobate	SBL231	Yes	#1	*Bolinopsis microptera*	100 %	99,69 %	XR_010160386.1	No
18S	Lobate	SBL231	Yes	#2	*Kiyohimea usagi*	100 %	99,69 %	MW647045.1	No
18S	Lobate	SBL231	Yes	#8	*Bolinopsis infundibulum*	100 %	99,69 %	MW647030.1	Yes
18S	Lobate	SBL232	Yes	#1	*Bolinopsis microptera*	100 %	99,69 %	XR_010160386.1	No
18S	Lobate	SBL232	Yes	#2	*Kiyohimea usagi*	100 %	99,69 %	MW647045.1	No
18S	Lobate	SBL232	Yes	#8	*Bolinopsis infundibulum*	100 %	99,69 %	MW647030.1	Yes
18S	Lobate	SBL234	Yes	#1	*Mnemiopsis* sp.	100 %	98,78 %	MF599331.1	Yes
18S	Lobate	SBL234	Yes	#2	*Mnemiopsis leidyi (Agassiz, 1865)*	100 %	98,78 %	MF599330.1	Yes
18S	Lobate	SBL234	Yes	#37	*Bolinopsis infundibulum*	100 %	98,78 %	MW647030.1	Yes
18S	Lobate	SBL245	Yes	#1	*Bolinopsis microptera*	100 %	100 %	XR_010160386.1	No
18S	Lobate	SBL245	Yes	#2	*Kiyohimea usagi*	100 %	100 %	MW647045.1	No

MARKER	MORPHOLOGICAL ID.	SAMPLE ID	BL	RESULT NUMBER	BLAST MATCH	QUERY	MATCH	AC NUMBER	SAME REGION
18S	Lobate	SBL245	Yes	#8	*Bolinopsis infundibulum*	100 %	100 %	MW647030.1	Yes
18S	Lobate	SBL250	Yes	#1	*Bolinopsis microptera*	100 %	99,60 %	XR_010160386.1	No
18S	Lobate	SBL250	Yes	#2	*Kiyohimea usagi*	100 %	99,60 %	MW647045.1	No
18S	Lobate	SBL250	Yes	#8	*Bolinopsis infundibulum*	100 %	99,60 %	MW647030.1	Yes
18S	Lobate	SBL264	Yes	#1	*Bolinopsis microptera*	100 %	99,69 %	XR_010160386.1	No
18S	Lobate	SBL264	Yes	#2	*Kiyohimea usagi*	100 %	99,69 %	MW647045.1	No
18S	Lobate	SBL264	Yes	#8	*Bolinopsis infundibulum*	100 %	99,69 %	MW647030.1	Yes
LCO	Euphausiid	SBL004	Yes	#1	*Hansarsia megalops (Sars, 1883)*	100 %	99,64 %	AY047603.1	Yes
LCO	Euphausiid	SBL004	Yes	#2	*Hansarsia megalops*	100 %	99,46 %	MG669402.1	Yes
LCO	Euphausiid	SBL017	Yes	#1	*Hansarsia megalops*	99 %	96,07 %	MT826936.1	Yes
LCO	Euphausiid	SBL017	Yes	#2	*Hansarsia megalops*	99 %	95,90 %	MT826940.1	Yes
LCO	Euphausiid	SBL018	Yes	#1	*Hansarsia megalops*	97 %	99,35 %	MG669402.1	Yes
LCO	Euphausiid	SBL018	Yes	#2	*Hansarsia megalops*	97 %	99,51 %	AY047603.1	Yes
SHA	Siphonophore	SBL123	No	#1	*Agalma clausi (Bedot, 1888)*	100 %	89,66 %	NC_080953.1	No
SHA	Siphonophore	SBL123	No	#2	*Agalma clausi*	98 %	89,49 %	AY935270.1	No
SHA	Hydrozoan	SBL127	No	#1	*Leuckartiara octona (Fleming, 1823)*	100 %	96,30 %	MG136743.1	Yes
SHA	Hydrozoan	SBL127	No	#2	*Leuckartiara octona*	99 %	96,30 %	MG136739.1	Yes
SHA	*Clytia* sp.	SBL247	Yes	#1	*Clytia* sp.	100 %	96,33 %	MK073056.1	Yes
SHA	*Clytia* sp.	SBL247	Yes	#2	*Clytia hemisphaerica (Linnaeus, 1767)*	100 %	96,33 %	MG811593.1	Yes
SHA	*Clytia* sp.	SBL251	Yes	#1	*Clytia* sp.	100 %	99,63 %	MF662616.1	Yes
SHA	*Clytia* sp.	SBL251	Yes	#2	*Clytia hemisphaerica*	100 %	99,63 %	KX665279.1	Yes
Zplank	*Metridia sp*.	SBL062	Yes	#1	*Chiridius armatus (Boeck, 1872)*	100 %	98,91 %	AY660604.1	Yes
Zplank	*Metridia sp*.	SBL062	Yes	#2	*Chiridius armatus*	100 %	99,13 %	AY660603.1	Yes
Zplank	*Metridia sp*.	SBL073	Yes	#1	*Thysanoessa raschii (M.Sars, 1863)*	100 %	100 %	KX675898.1	Yes
Zplank	*Metridia* sp.	SBL073	Yes	#2	*Thysanoessa raschii*	100 %	100 %	KX675897.1	Yes

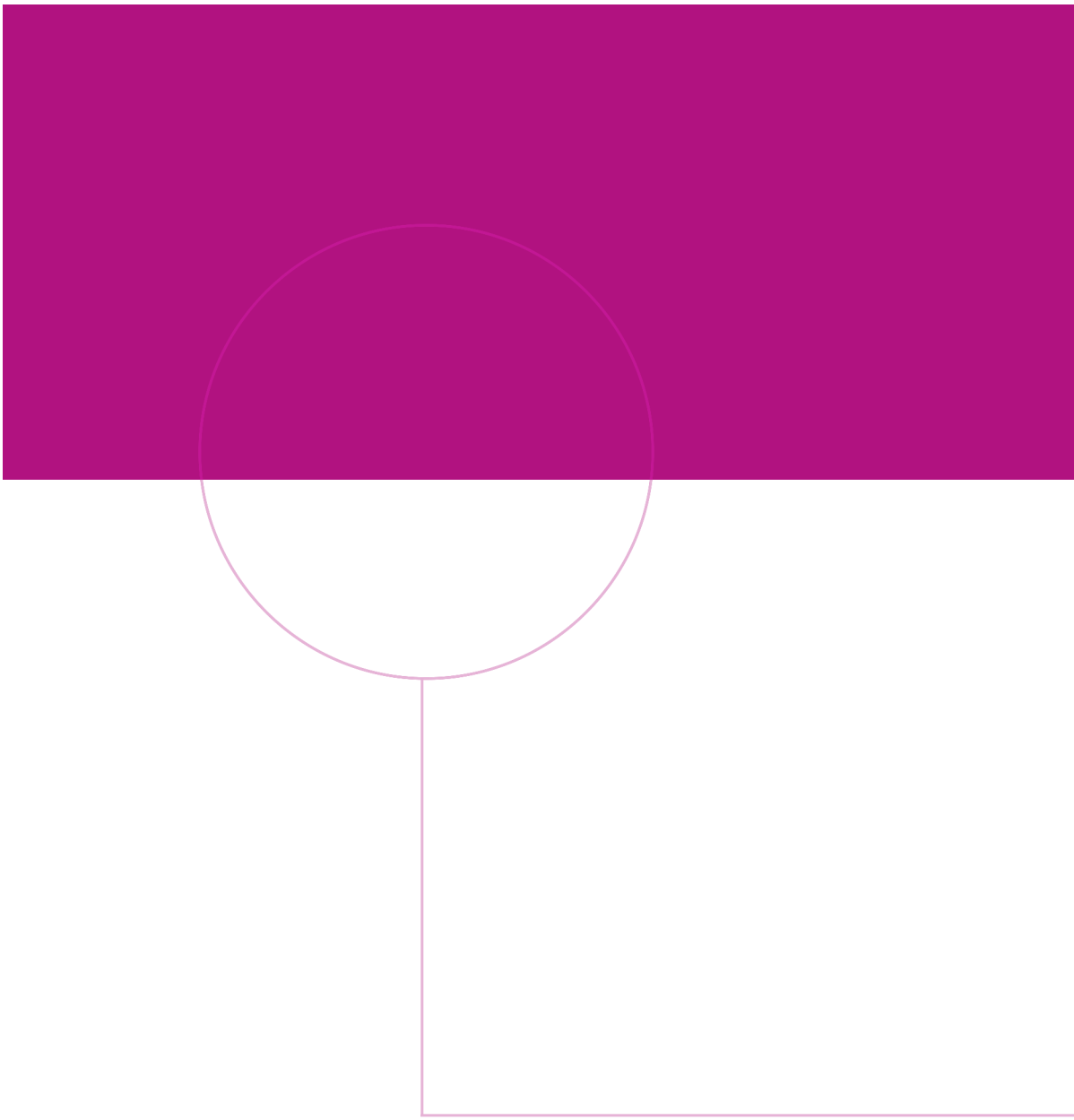
Appendix B: taxonomy of specimens caught during fieldwok for spectral measurements. They are sorted alphabetically from the highest classification. The lowest certain taxonomic level is included, and the author is described if relevant.

KINGDOM	PHYLUM	CLASS	ORDER	FAMILY	GENUS	SPECIES	AUTHOR
Animalia	Annelida	Polychaeta	Phyllodocida	Tomopteridae	*Tomopteris* spp.		Eschscholtz, 1825
Animalia	Arthropoda	Copepoda	Calanoid	Aetideidae	*Aetideopsis*	*armata*	Boeck, 1872
Animalia	Arthropoda	Copepoda	Calanoida	Metridinidae	*Metridia* spp.		
Animalia	Arthropoda	Malacostraca	Euphausiacea	Euphausiidae	*Hansarsia*	*megalops*	G.O. Sars, 1883
Animalia	Arthropoda	Malacostraca	Euphausiacea	Euphausiidae	*Thysanoessa*	*raschii*	M. Sars, 1864
Animalia	Cnidaria	Hydrozoa	Anthoathecata	Pandeidae	*Leuckartiara*	*octona*	Fleming, 1823
Animalia	Cnidaria	Hydrozoa	Leptophecata	Campanulariidae	*Clytia* spp.		Lamouroux, 1812
Animalia	Cnidaria	Hydrozoa	Leptophecata	Tiarannidae	*Modeeria*	*rotunda*	Quoy & Gaimard, 1827
Animalia	Cnidaria	Hydrozoa	Siphonophorae indt.				
Animalia	Cnidaria	Scyphozoa	Semaeostomeae	Ulmariidae	*Aurelia*	*aurita*	Linnaeus, 1758
Animalia	Ctenophora	Nuda	Beroida	Beroidae	*Beroe* spp.		Gronov (ex Browne, 1756) 1760
Animalia	Ctenophora	Tentacula	Cydippida	Euplokamidae	*Euplokamis*	cf. *dunlapae*	Mills, 1987
Animalia	Ctenophora	Tentacula	Cydippida	Mertensiidae	*Mertensia*	ovum	O. Fabricius, 1780
Animalia	Ctenophora	Tentacula	Cydippida	Pleurobrachiidae	*Pleurobrachia*	pileus	O.F. Müller, 1776
Animalia	Ctenophora	Tentacula	Lobata	Bolinopsidae	*Bolinopsis*	*infundibulum*	O.F. Müller, 1776
Animalia	Ctenophora	Tentacula	Lobata indt.				

Appendix C: Comparison of the spectra found in this study and literature. Included is taxa described in the study, ID method; how the respective papers identified their specimens. Method; information about how the studies measured or/and stimulated bioluminescence. λ_{max} and FWHM measured in nm, any deviation available is included. Depth the specimens were caught at. Period of year, location specimens were found, information about them, number found (n), and source. There was no information on stimuli success. * indicates that the spectra is the same as in this study. OMA= optical multichannel analyzer. M= western Mediterranean,P= 100 miles west of Point Conception (eastern temperate of specimens examined) ,G= Gulf of Maine S= Santa Barbara Channel.

TAXA	ID METHOD	METHOD	λ_{max} (nm)	FWHM (nm)	DEPTH (m)	TIME	LOCATION	SPECIMEN INFO	(n)	SOURCE
Tomopteris spp.	Morphology	Spectrometer, Ocean Optics QE65000 with attached fiber optics.	565		Often 400 m, but between 269 and 1216 m.	1999-2011	Monterey Bay, USA	Good condition		Francis *et al*. (2014)
T. helgolandica	Morphological, descriptions from different authors	Digital picture	573 ±2,0 SEM	130 ±1,0 SEM	0-250	2011-2016	Fjord in West Norway	Anaesthesized before experiement	3	Gouveneaux *et al*. (2017)
T. helgolandica	Morphological, descriptions from different authors	Live spectra	573	51	0-250	2011-2016	Fjord in West Norway	Anaesthesized before experiement	1	Gouveneaux *et al*. (2017)
T. planktonis	Morphological, descriptions from different authors	Digital picture	450 ±0,5 SEM	23 ±1,0	0-180	nov.14	West Norway	Anaesthesized before experiement	3	Gouveneaux *et al*. (2017)
Metridia longa	Morphological?	AMINCO luminescent spectrometer	480		50-100		White sea	Stage IV–VI of development (0.3–1.5mm in size)		Markova *et al*. (2004)
Hansarsia megalops (Nematoscelis megalops)		In vitro or homogenate reaction	470	48		1976		2		Herring (1983)
Metridia lucens		Spectrophoto-fluorometer to measure. Mechanical and electrical stimuli	482	85	0-30 m (plankton net)	July and August, 1960	Cape Cod Bay, USA	Placed on ice then crushed to make the flash last longer	40 were found	David and Connover (1961)
Thysanoessa raschii		Spontaneous or chemically stimulated	476	-						Boden and Kampa (1959)

TAXA	ID METHOD	METHOD	λ_{max} (nm)	FWHM (nm)	DEPTH (m)	MONTH/ PERIOD/ YEAR	LOCATION	SPECIMEN INFO	(N)	SOURCE
Clytia hemisphericum	Morphological, descriptions from different authors	OMA	504**	37	20	April	M	Well preserved specimens	1	Haddock and Case (1999)
Beroe abyssicola	Morphological, descriptions from different authors	OMA	491 ± 1.1 SE*	88**	Deep, 489		P S	Well preserved specimens	3	Haddock and Case (1999)
Beroe cucumis	Morphological, descriptions from different authors	OMA	489 ± 4.7 SE	88*	Shallow, 37		G M S	Well preserved specimens	11	Haddock and Case (1999)
Beroe cucumis	Morphological? Just said they were identified.	OMA	484	83	Zp.net	1982-1983	California		1	Widder (1983)
Beroe cucumis		OMA: mechanical or electricity	479, b=496	94	0-125	April	Sargasso Sea		1?	Latz *et al*. (1988)
Beroe gracilis	Morphological, descriptions from different authors	OMA	495	89	Shallow, 12	January to July, 1990-1992	Santa Barbara Channel	Well preserved specimens	2	Haddock and Case (1999)
Bolinopsis infundibulum			505							Herring 1983, source within
Bolinopsis infundibulum	Morphological, descriptions from different authors	OMA	488 ±4.2 SE	88*	100		G M S	Well preserved specimens	8	Haddock and Case (1999)
Bolinopsis sp.		OMA: mechanical or electricity	488	80	0-125	April	Sargasso Sea		1?	Latz *et al*. (1988)
Euplokamis sp.	Morphological, descriptions from different authors	OMA	483 ±1,9 SE	85	243	August	G	Well preserved specimens	4	Haddoc case 1999
Euplokamis sp.	Morphological, descriptions from different authors	OMA	483 ±1,9 SE	85	243	August	G	Well preserved specimens	4	Haddoc case 1999



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