THE REGULATION AND ORIGIN OF BIOLUMINESCENCE IN THE HYDROID *OBELIA*

Valerie Jane Morse

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Cardiff University

School of Pharmacy and Pharmaceutical Sciences

Redwood Building

King Edward VII Avenue

Cardiff CF10 3NB

"For see, the glow-worm lights her amorous fire!

Thus, ere night's veil had half obscured the sky,

The impatient damsel hung her lamp on high:

True to her signal, by love's meteor led,

Leander hasten'd to his Hero's bed."

The Rev Gilbert White, 1902.

Summary

There are many questions associated with the understanding of the origin and regulation of bioluminescence. A key question underlying this thesis was why coelenterazine evolved as the most common marine luciferin. Another problem addressed was the coelenterazine source of the bioluminescent hydroid *Obelia*, which is unknown. Coelenterazine is an integral part of Obelia's photoprotein, triggered by Ca^{2+}. The thesis also investigated the uncertainty over how Ca^{2+} enters *Obelia's* photocytes. *Obelia* is considered to have four species, but they have been misidentified. Green fluorescent protein (GFP) acts as a fluor in *Obelia* producing green light. The selective advantage of this green bioluminescence requires confirmation.

Results showed that *Obelia geniculata* cultures rapidly lost their bioluminescence, indicating *Obelia* requires a dietary supply of coelenterazine. Adding coelenterazine to *Obelia* briefly restored its bioluminescence. Levels of obelin dropped in *Obelia geniculata* cultures. *Obelia longissima* had a lower level of coelenterazine and bioluminescence than *Obelia geniculata*. Coelenterazine was detected in *species* living on *Obelia* and, three non luminous species of copepod. A range of species were identified in zooplankton, as possible coelenterazine sources. Ratios of obelin to apoobelin changed in older colonies. The problem of misidentification of *Obelia* species was solved by utilising the fluorescent patterns of *Obelia's* photocytes.

GFP was recorded for the first time in the hydrotheca and tentacles of *Obelia dichotoma*. Fluorescence maxima for *Obelia geniculata* and *Obelia dichotoma*

were different. GFP in *Obelia* was found to photobleach far slower than GFP in EGFP. This supports the hypothesis that *Obelia* has a molecular mechanism which protects its GFP from photobleaching.

Light emission from *Obelia geniculata* was different from *Obelia longissima*. This suggests that the mode of entry of Ca^{2+} into the photocytes of these two species is different. To investigate the exact pathway by which initial stimulation of *Obelia* causes Ca^{2+} to enter the photocytes, experiments were conducted using K^+ channel blockers. The potassium ion channel blockers tetraethyl ammonium chloride and 4-aminopyridine both produced a bioluminescent response in *Obelia geniculata*. This suggested that the pathway included K^+ channels.

In a polar solvent coelenterazine produced low chemiluminescence, which increased with increasing luciferin concentration. Human albumin and BSA increased this effect. This supported the solvent cage hypothesis that bioluminescent proteins originally evolved as primitive oxygenase enzymes.

DECLARATION

This work has not been submitted in substance for any other degree or award at this or any other university or place of learning, nor is being submitted concurrently in candidature for any degree or other award.

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STATEMENT 1

This thesis is being submitted in partial fulfilment of the requirements for the degree of PhD

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This thesis is the result of my own independent work/investigation, except where otherwise stated.

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CONTENTS

CHAPTER 1 – Introduction

CHAPTER 2 – Materials and Methods

CHAPTER 3 – Identification and distribution of *Obelia*

CHAPTER 4 – How is light emitted in *Obelia*?

CHAPTER 5 – How is coelenterazine obtained by *Obelia*?

CHAPTER 6 - The effect of GFP on the wavelength of light emitted by Obelia

CHAPTER 7 – Evolution of bioluminescence

CHAPTER 8 – Bioluminescence in education

CHAPTER 9 – General discussion

Appendices   307

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Dedication

This thesis is dedicated to my mother, Marguerita who tragically died young. Her dream was for me to study at University. Memories of her have spurred me on.

List of figures

List of tables

Abbreviations

ADP   Adenosine diphosphate

AMP   Adenosine monophosphate

ATP   Adenosine triphosphate

BSA   Bovine serum albumin

CL cts/s Chemiluminescent counts per second

CL cts/10s Chemiluminescent counts per 10 seconds

EDTA   Ethylene diamine tetra acetic acid

EGTA   Ethylene glycol-bis (2-aminoethylether)-N, N, N^1 N^1-tetraaceticacid

FLIM   Fluorescence Life-time Imaging Microscopy

GFP   Green fluorescent protein

GPS   Global positioning system

HEPES N-[2-hydroxyethranosideyl] piperazine-N' [2-ethanesulfonic acid]

IPTG   Isopropylβ-D-thiogalactopyranoside

MHPA Milford Haven Port Authority

mM   millimolar

mol   moles

MTL   mean tidal level

OAP   Odd area profile

OAS   Odd area set

ppT   parts per Thousand

nmol   nanomole

pmol   picomole

µl   microlitre

µM   micromolar

RT   room temperature

$t_{1/2}$ half-life

TE   Tris-EDTA

TEA   Tetra ethyl ammonium

s   second

SEM   standard error of the mean

w/v   weight to volume

Chapter one

Introduction

Introduction

Bioluminescence is the emission of light from living organisms (Harvey, 1957). This thesis is focused on the bioluminescent hydroid *Obelia,* in particular the three *Obelia* species found in the Milford Haven waterway in Pembrokeshire i.e. *Obelia geniculata, Obelia dichotoma* and *Obelia longissima.* This thesis includes an investigation into whether bioluminescence and fluorescence can be utilised to accurately distinguish between the 3 species which are often confused. *Obelia* is found beyond the tide line of the entire British coast and is practically cosmopolitan occurring in coastal waters throughout the world.This study of *Obelia,* had the overall aims of investigating how it regulates its light emission and what the origin of its bioluminescence is.

1.1 Marine bioluminescence

The majority of light in the terrestrial world is emitted from incandescent sources such as the sun. On earth light and colour are utilised by nature to attract mates, lure prey, and scare predators and to provide a means of camouflage. Only a small percentage of terrestrial light is produced by bioluminescence i.e. light produced by living organisms e.g. light produced by the glow-worm *Lampyris noctiluca* and the firefly *Photinus pyralis*. But in the dark depths of the oceans very little sunlight filters down, and hence marine creatures communicate mainly by using bioluminescence. "Flashes and trails provide information on potential partners, predators, meals and mates. Anything that can be done with colour in a sunlit environment can be done with light in the dark" (Herring, 1990).

1.1.1 Bioluminescence and chemiluminescence

One of the first observations which Charles Darwin made during his five year voyage on the Beagle (1831-1836) concerned a fascinating display of bioluminescence. 'The sea was luminous in specks & in the wake of the vessel, of a uniform, slightly milky colour. – When the water was put into a bottle, it gave out sparks for some minutes after having been drawn up. – When examined both at night and next morning, it was found full of numerous small (but many bits visible to the naked eye) irregular pieces of (a gelatinous?) matter. The sea next morning was in the same place equally impure'. This was most probably dinoflagellate bioluminescence (Darwin, 1859; Vassel et al. 2012). Eilhardt Weidemann (1852-1928) investigated the different mechanisms by which light can be produced in 1888. He distinguished between incandescence where a chemical reaction produces both light and heat, and what he termed "cold light". "Cold Light" is light production from chemiluminescence. Here the chemical reaction produces energy to allow electrons to be excited to higher energy levels. Chemiluminescence always involves chemical oxidation and no energy is lost as heat. "The secret in chemiluminescence is that the key reaction intermediate must generate its energy very rapidly so that the electron can be excited, before energy is lost as heat" (Campbell, 2003b). Bioluminescence is the emission of light from living organisms. It is believed that E. Newton Harvey (1887 to 1959) was the first person to use the term bioluminescence to describe luminescence from living organisms, in his paper on the mechanism of light production in animals (Harvey, 1916; Shimomura, 2006). All bioluminescent light is produced by chemiluminescence (Campbell, 1994).

1.1.2 Photoluminescence

In different types of luminescence the energy source for the excitation of electrons varies. Both fluorescence and bioluminescence are exhibited by the coastal hydroid *Obelia geniculata* and it is important to explain how fluorescence differs from bioluminescence. Both fluorescence and phosphorescence are types of photoluminescence. In photoluminescence the energy to promote electrons to higher energy levels comes from light (Campbell, 1994).

Fluorescence as produced by green fluorescent protein (GFP) is a type of photoluminescence. This is short lived emission and requires energy to produce the electronically excited states. This energy is supplied by "the absorption of electromagnetic radiation in the ultra violet, visible or infra red regions" (Campbell, 1988). True phosphorescence is also a type of photoluminescence but it is long-lived emission. Energy for its electronically excited state is provided by absorption of UV light. In phosphorescence there is intersystem crossing between singlet and triplet spin states (refer to Fig 1.1). Unfortunately the term phosphorescence is often used incorrectly by the media and this can lead to confusion (Campbell, 1988).

1.1.3 The components of a chemiluminescent reaction.

It has now been established that there are three key components of a chemiluminescent reaction (refer to Fig 1.2). The first is a luciferin molecule that needs to be oxidised to produce light e.g. coelenterazine. Secondly a luciferase is required that acts as a protein catalyst and thirdly oxygen is also needed. During the reaction the luciferin is oxidised and the end products of the reaction are

oxyluciferin, light and carbon dioxide. In addition to these three key components cofactors may also be involved. Typical cofactors include ATP, cations e.g. calcium ions and fluors such as GFP. More recently the existence of photoproteins in some marine bioluminescent systems has been established. In photoproteins the luciferase is an apoprotein e.g. apoobelin in the luminescence of *Obelia geniculata (*Linnaeus*)* and apoaequorin in *Aequorea aequorea* (Campbell & Herring, 1990).
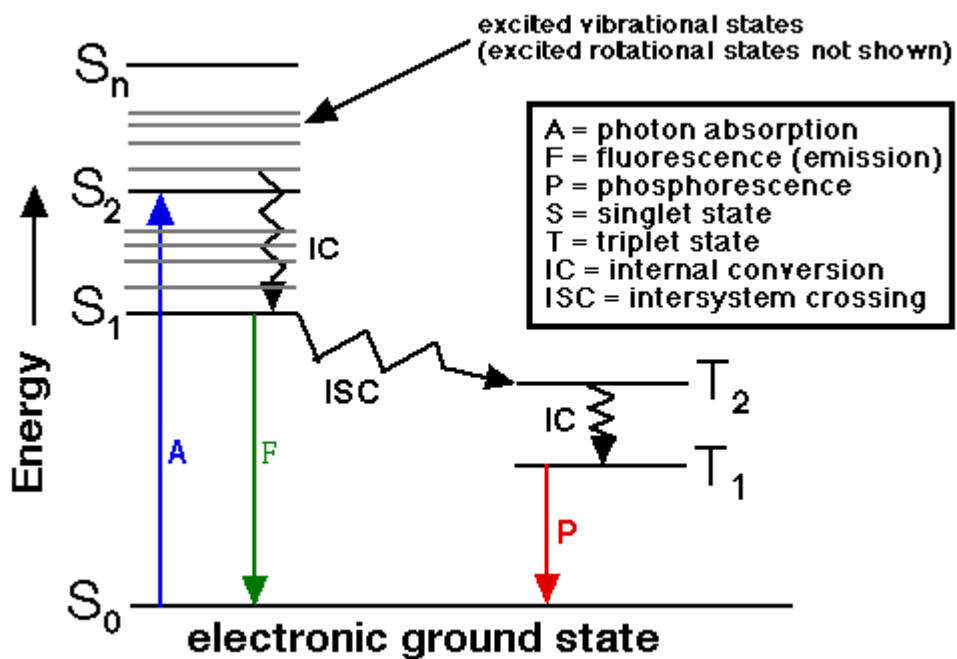
Fig.1.1.A Jablonski diagram. The diagram illustrates that electrons can return to the ground state by a number of different routes. http://www.shsu.edu/~chm-tgc/chemilumdir. Author Dr TG Chasteen. Accessed 2.4.12. 7.15am.

$$\boxed{Luciferase}$$

$$Luciferin + O_2 + cofactors \longrightarrow oxyluciferin + light + CO_2$$

Fig.1.2. The key components of a chemiluminescent reaction. In a classic luciferin- luciferase reaction the luciferin is oxidised, with the luciferase acting as a catalyst.

1.1.4 The early history of bioluminescence

The bioluminescence of glow-worms and fireflies is recorded in the Chinese shih Ching (book of odes). It is believed to date from 1500 to 1000 BC. The Chinese also observed phosphorescence in the sea, but at this time had no microscopes or lenses to investigate its cause. Early in Japanese history firefly collecting was a popular past time (Harvey, 1957). Aniximenes as early as 500BC described the light exhibited by the sea when struck with an oar (Harvey, 1952). Aristotle's (384 to 322 BC) observations of nature included a study of many marine creatures (Weeks, 1992; Wilson & Hastings, 2013). His writings on light and colour in *De Anima* seem to suggest that he was aware of luminescence from dead fish and flesh. He observed many different marine organisms when recording observations for *De Anima*, working on the shore of a lagoon on the Greek island of Lesbos. Aristotle recognised that "some things" could produce cold light (Harvey, 1957). Pliny (AD23 to 79) gave more specific descriptions of luminescence. In *Historia naturalis* he referred to glow-worms, *Pholas* the luminous boring mollusc and lantern fish. *Historia naturalis* was translated into English by Philemon Holland (Campbell, 1994). Pliny also famously recorded a jellyfish with a luminous slime "Plumo marinus". He explained how the slime could be used as a torch "a walking stick rubbed with Plumo marinus will light the way like a torch" (Harvey, 1952). He was probably describing *Pelagia notiluca* a luminous scyphozoan (Shimomura, 2006). Around 1600 a new rational approach to learning was inaugurated by Francis Bacon, an English philosopher; this is now recognised as a key period in science. This new approach saw science become clearly divided from magic. In 1605 Bacon also recorded how drops of sea water "struck off by

the motion of the oars in rowing, seem sparkling and luminous" (Harvey, 1952).

Robert Boyle (1626 to1691) performed a classic experiment when investigating samples of glowing wood and glowing flesh. We now know that the fungus *Armillaria mellea* causes wood to luminesce and bioluminescent bacteria create the phenomenon of glowing flesh. Boyle used a bell jar in which he created a vacuum by utilising an air pump. He discovered that when air was removed from the bell jar the glowing light ceased. "This was the first demonstration of oxygen requirement in bioluminescent reactions, some 100 years before Priestley and Scheele discovered it" (Campbell, 1994). Boyle also observed a difference in the light from burning coal compared to that from glowing wood "live coal was actually and vehemently hot". Whereas he observed that the glowing wood was "not sensibly lukewarm" (Campbell, 1988).

"Raso ligno, pareum adeo in tenebris splendet", is how Forskål (1732–1763) described the bioluminescence of the jellyfish he called *Medusa aequorea* in 1775. This roughly translates as "struck by wood to such an extent it shines brightly in the darkness" (Campbell, 1988). Forskål was the first person to name *Medusa aequorea* (Shimomura, 2006). The same luminous medusa was renamed *Aequorea Forskalea* (Peron & Lesueur, 1809) and this name is still used in the UK. However in the USA scientists refer to it as either *Aequorea aequorea* (Murbach and Shearer) or *Aequorea victoria (*Shimomura, 2006*)*. These three names are used for what is probably the same species and can create a great deal of confusion. In this thesis I will use the term *Aequorea aequorea* as commonly used by Shimomura.

Forskål could not have foreseen that in the 21st century the photoprotein and green fluorescent protein (GFP) extracted from this luminous medusa would be used as important tools in many medical applications.

1.1.5 The biochemistry of marine bioluminescence

It was not until the 1880's that Raphael Dubois (1849-1929) carried out important experiments on two luminous species the beetle *Pyrophorus* and the bivalve *Pholas dactylus* to discover two of the key components "that were needed for burning without fire" (Campbell, 1994; Shimomura, 2006). He named the components luciferin and luciferase and these terms are still used today in the study of chemiluminescent and bioluminescent reactions. He established that the luciferin was heat stable but the luciferase was heat sensitive. This led to Dubois publishing two key papers. The first on the luminous click beetle was published in 1885 followed by the second on *Pholas dactylus* in 1887. We now know that all luciferases are enzymes and unique to the luminous organism they occur in (Campbell & Herring, 1990). E. Newton Harvey (1887 - 1959) was inspired by Dubois's work and went onto study bioluminescence in a wide variety of organisms (Campbell, 1988). He specifically looked for the existence of the luciferin-luciferase system in each luminous creature he studied (Cormier, 1978).

In 1957 after some painstaking experiments Osamu Shimomura isolated the Vargula-type luciferin from *Vargula hilgendorfii.* It can be argued that an even more notable discovery was made by Shimomura at Friday Harbour laboratory USA in 1962. Working with Johnson and other colleagues he isolated a protein from the luminous jellyfish *Aequorea aequorea.* This protein aequorin flashed when calcium was added to it. In 1966 he discovered a similar protein in the

parchment tubeworm *Chaetopterus* which led to the concept of photoproteins being established in 1966 (Shimomura, 2006). The DNA for aequorin, which we now know to be a photoprotein, was cloned in 1985. It consists of 189 amino acids with 3 calcium binding sites (Campbell, 1994). In all luminous coelenterates the luciferin is coelenterazine (an imidazolopyrazine). In the jellyfish *Aequorea* and the hydroid *Obelia* the protein catalyst (the luciferase) binds the coelenterazine and oxygen so tightly that it can be extracted as a photoprotein complex. It has now been established that within the aequorin photoprotein complex coelenterazine is covalently bonded to apoaequorin. As soon as calcium ions are added the photoprotein flashes rapidly (Campbell, 2003b).
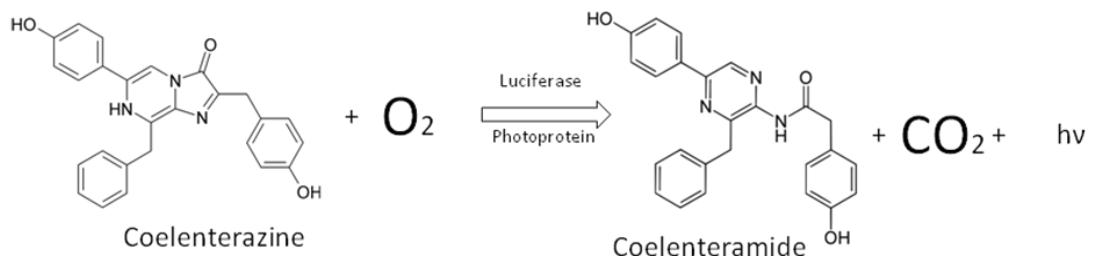
Fig.1.3. The reaction of coelenterazine. Coelenterazine covalently bound to apoaequorin exists within a photoprotein e.g. aequorin. It is triggered by calcium ions to produce light, carbon dioixide and colenteramide (Campbell, 2003b).

Scientists have gone on to discover that different photoproteins exist in some luminous marine species e.g. obelin in species of *Obelia* and clytin in *Clytia hemisphaereica*. Conversely other luminous marine organisms have a luciferin luciferase system which do not involve photoproteins e.g. *Renilla* (the sea pansy).

Significant discoveries were made in the 1960's about the chemical reactions involved in marine bioluminescent systems. The bioluminescence of the jellyfish *Aequorea* was studied at Princeton University by Frank. H. Johnson's research group simultaneously Dr Milton Cormier's group worked on the sea pansy *Renilla* at the University of Georgia. A tiny sample of coelenterazine was isolated by Cormier from *Renilla* in the mid 1960's but at the time its chemical identity was unknown (Hori & Cormier, 1965). In 1974 Shimomura predicted that coelenterazine was contained within aequorin (Shimomura et al. 1973b). It was isolated in 1975 by Inoue et al from *Watasenia scintillans* a luminous squid (Inoue et al. 1975).

Using synthetic coelenterazine Shimomura and Johnson were able to establish that coelenterazine was part of the aequorin molecule and that active aequorin can be regenerated from coelenterazine (Shimomura & Johnson, 1975b). This regeneration forms the basis of the coelenterazine assay (Campbell, 2003(a)).

From the late 1970's onwards researchers began to realise that coelenterazine is the luciferin in a wide range of bioluminescent marine organisms including *Aequorea, Renilla, Obelia geniculata, Cavernularia* (sea cactus*),* and *Ptilosarcus* commonly called the seapen (Shimomura et al. 1980; Campbell & Herring, 1990). Many different names were proposed for this ubiquitous marine luciferin including *Watasenia* preluciferin and *Renilla* luciferin. However the one word coelenterazine is now the most commonly used throughout the scientific community (Shimomura, 2006).

From the work of a variety of scientists including Shimomura and Inoue we now know far more about the biochemistry of photoproteins inside luminous marine organisms. Aequorin reacts rapidly with calcium ions emitting light at a maximum of 460 to 465 nm. In the process aequorin is decomposed into coelenteramide; apoaequorin and CO_2 (refer to Fig.1.3.) If sufficient coelenterazine is present, it reacts with apoaequorin slowly reforming the photoprotein aequorin (Shimomura, 2006). Shimomura has isolated eight different types of aequorin (iso enzymes) from *Aequorea aequorea* (Shimomura, 1986).

Aequorea aequorea's bioluminescence arises from aequorin found in specialised photocytes in the jellyfish's umbrella. In 1963 Johnson and colleagues noted that the bioluminescence from *Aequorea aequorea* was slightly greener than that produced by Ca^{2+} stimulation of the isolated aequorin. Similar "spectral shifts" were observed by Morin and Hastings in luminous coelenterates including *Obelia geniculata* (Morin & Hastings, 1971b). Ward and Cormier explained these spectral differences when they investigated the luminescent sea pansy *Renilla* (Ward & Cormier, 1979). The difference is due to the presence of a green fluorescent protein (GFP). Energy from the excited-state molecule of the luminescent reaction (e.g. coelenteramide) is transferred to GFP which becomes the light emitter (Campbell, 1988). As part of this process GFP alters the wavelength of the light making it greener.

1.1.6 The occurrence of bioluminescence

Bioluminescence can be referred to as living light. Some terrestrial species exhibit bioluminescence on land, the most familiar being the glow-worm and firefly.

There are luminous fungi but no luminous plants. Bioluminescence is most common in the sea where in the cold depths of the oceans most organisms are luminous. In the 1800's most zoologists doubted the existence of life at great depths. Edward Forbes in 1841 obtained specimens at 230 fathoms, but thought that life could not exist beyond 300 fathoms (Harvey, 1957). Charles Darwin had no facilities for deep sea collection during the voyage of HMS Beagle. Charles Darwin writing in his famous "origin of the species" recognised as early as 1868 that the evolution of luminescence in nature could not easily be explained.

" The luminous organs which occur in a few insects, belonging to widely different families, and which are situated in different parts of the body, offer under our present state of ignorance, difficulty almost parallel with that of electric organs" (Darwin, 1868).

In 1952 Newton Harvey highlighted the fact that there is no clear pattern to the evolution of luminous species in phyla. "It is as if the main groups of organisms are listed on a blackboard, and a handful of sand is thrown. The luminous species are where the sand sticks" (Harvey, 1952; Campbell, 2003a; Vassel et al. 2012). Since Harvey's time more examples of luminous marine organisms have been discovered but, a pattern in its occurrence has still not emerged. Peter Herring has stated "The occurrence and known chemistry of bioluminescence exhibits no obvious biological pattern in different groups of organisms" (Herring, 1978). More recently scientists have been able to use sophisticated research vessels such as RRS Discovery and submersibles e.g. "Alvin" to discover that bioluminescent species occur from the ocean's surface right down through the depths to the ocean floor (Campbell, 1988). Bioluminescence occurs in some 700 genera representing

18 phyla and plays an important role in the ecology of the sea. In 1987 Herring published an extensive list of the genera of organisms known to contain luminous species (Herring, 1987; Thomson et al. 1997; Shimomura, 2006; Vassel et al. 2012). Table 1.1 gives examples of bioluminescent marine organisms from a range of phyla (Campbell, 1988). In Chapter seven of this thesis there is an investigation to discover if bioluminescent proteins such as coelenterazine evolved as primitive mono oxygenase enzymes, thus testing the "solvent cage hypothesis". This may help to answer the key question as to why coelenterazine bioluminescence evolved independently in so many different marine species (Vassal et al. 2012). "It has been estimated that present day luminous organisms come from as many as 30 different evolutionary distinct origins" (Hastings, 1983; Hastings, 1995; Hastings & Morin, 1998).

Phylum	Class	Order	Family	Species
Sarcomastigophora	Actinopoda	Radolaria	Thalassicollidae	*Thallassicolla*
Cnidarian	Hydrozoa	Thecata	Campanulariidae	*Obelia geniculata*
Cnidarian	Hydrozoa	Thecata	Campanulariidae	*Clytia hemisphaerica*
Cnidarian	Scyphozoa	Semaeostomea	Pelagiidae	*Pelagia noctiluca*
Cnidarian	Anthozoa	Pennatulacean	Renillidae	*Renilla reniformis*
Ctenophora	Tentaculata	Lobata	Mnemiopsidae	*Mnemiopsis leidyi*
Mollusca	Cephalopoda	Teuthoidea	Enoploteuthidae	*Watasenia scintillans*
Mollusca	Bivalvia	Myoida	Pholadidae	*Pholas dactylus*
Arthropoda	Crustacea	Myodocopida	Cypridinidae	*Vargula hilgendorfii*
Echinodermata	Ophiuroidea	Ophiuroid	Amphiurdae	*Amphipholis squamata*
Chordata	Thalicea	Pyrosomida	Pyrosomitidae	*Pyrosoma*
Chordata	Osteichthyses	Salmoniformes	Sternoptychidae	*Argyropelecus*

Table.1.1. Examples of luminous marine organisms, from a range of Phyla. Adapted from Campbell, 1988.

1.2 The five classes of luciferins

Following more recent scientific research five classes of luciferin have now been identified: aldehydes, imidazolopyrazines, benzothiazole, linear tetrapyrroles and flavins (Campbell & Herring, 1990). An aldehyde luciferin is used by the luminous limpet *Latia* whereas benzothiazoles are the luciferin in luminous beetle such as the female glow-worm. Luciferins with a tetrapyrole structure have been characterised in dinoflagellates, euphasids and the dragon fish *Malacosteidae*. But the most commonly utilised luciferins in the marine environment are the imidazolopyrazines, which include coelenterazine.

1.2.1 Imidazolopyrazines

The vast majority of bioluminescent organisms exist in the oceans at a variety of depths. Most of this bioluminescence involves an imidazolopyrazine based luciferin (Campbell & Herring, 1990). "Therefore a key question is, how are imidazolopyrazines synthesised and by which organisms?" (Thomson et al. 1995).

Two types of luciferin have the structure of an imidazolopyrazine. The first to be discovered was *Vargula* type luciferin extracted from the sea fire fly *Vargula hilgendofii.* This luminous ostracod is also called *Cypridina* and there is a complex continuing debate about its name. The *Vargula*-type luciferin has a "molecular weight of 478 Daltons" (Inouye et al. 1969). Shimomura was the first scientist to crystallise this luciferin (Shimomura, 2006). So far a *Vargula*-type luciferin has only been identified in two phyla Arthropoda and Chordata (Thomson et al. 1995).
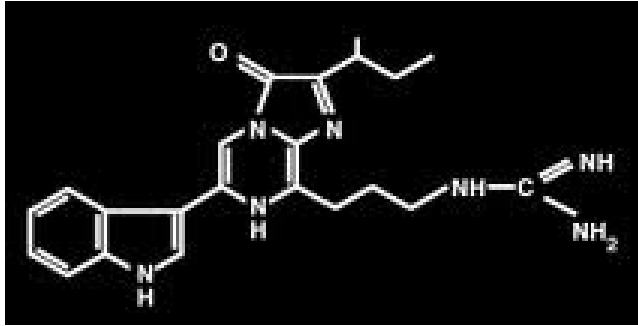
Fig.1.4. The structure of *Vargula*-type luciferin.
http://www.lifesci.ucsb.edu/~biolumi.

However the majority of imidazolopyrazine bioluminescence is the result of chemiluminescent reactions where coelenterazine is the luciferin. The name coelenterazine originates from the historic term for Cnidarians "coelenterates" (Shimomura, 2006).
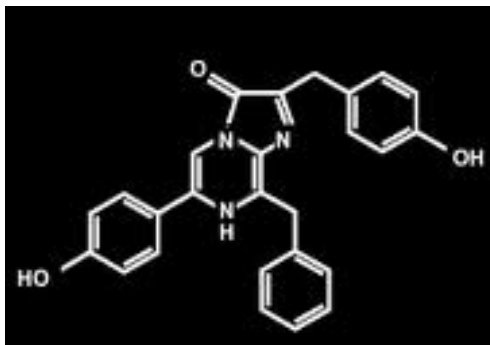
Fig.1.5. The structure of coelenterazine. http://www.lifesci.ucsb.edu/~biolumi. Author Arizona State University.Accessed 08.04.2011 8.23am.

Some marine organisms use coelenterazine in a classic luciferin luciferase reaction as originally investigated by Dubois (Weeks, 1992; Campbell, 1994).Whereas other marine organisms contain photoproteins which utilise coelenterazine bound in a complex with apoprotein and oxygen (Weeks, 1992; Shimomura, 2006). As a result of research by many different scientists it has now been established that

coelenterazine is the luciferin used by organisms in eight different marine phyla: - Cnidarians, Ctenophore, Mollusca, Protozoa, Echinodermata, Arthropoda, Chaetognatha and Chordata (Thomson et al. 1997; Vassel et al. 2012). Significant levels of coelenterazine have also been found in some species of non luminous marine organisms (Campbell, 2003a; Shimomura, 2006). Imidazolopyrazine luciferins can emit light in the violet to blue-green range of the spectrum. Coelenterazine has only been discovered as a luciferin in marine bioluminescence (Shimomura, 2006; Haddock et al. 2010). No example of a terrestrial animal using coelenterazine has ever been found. Bioluminescent coelenterates, decapods, squid, copepods, radolarians, some fish and ostracods have all been found to use imidazolopyrazine luciferins (Campbell, 2003(a)).

1.3 Coelenterate bioluminescence

1.3.1 Bioluminescent Zoophytes

Hydroids were originally classified as Zoophytes. This is a term historically used to describe hydroids and other marine colonial animals with the "overall growth-form of a plant" (Cornelius, 1995). Johnston in his book of British zoophytes (Johnston, 1847) explains how the term included all the organisms classified as Anthozoans including hydroids, sponges and corals. He states that zoophyte was first used to describe organisms that could not be easily classified into either the plant or animal kingdoms. Hydroids were included in the zoophytes due to their "middle nature" i.e. they had some characteristics of plants but other animal like characteristics. Johnston (Johnston, 1847) quotes a short poem by Crabbe which

reflects the difficulties of classifying hydroids and refers to the planula's role in their life cycle.

 "Involved in sea-wrack, here you find a race,

Which science doubting, knows not where to place;

On shell or stone is dropped the embryo seed,

And quickly vegetates a vital breed."

Johnston disagreed with naturalists who used the term more widely to describe any marine organism with polyps, but also sea–urchins, sea-figs, jellyfish and star fish. He limited the term to include any marine organism with polyps that was "invertebrate, inarticulate, soft, irritable and contractile".

Interestingly Johnston also comments on how human physiologists can learn from studying less complex marine life forms, such as zoophytes (Johnston, 1847). This alludes to the concept of marine model systems which has in the 20th and 21st centuries led to many vital discoveries in science e.g. key features of the nervous impulse were discovered by studying the giant axons in squid (Hodgkin and Huxley, 1952). Zoophytes are now classified as Cnidarians. Bioluminescent Cnidarians are reviewed in section 1.3.2. Key examples of bioluminescent Cnidarians which utilise coelenterazine are included. Bioluminescent Ctenophores are reviewed in section 1.3.3 as they provide a useful contrast, in that they use photoproteins, but no GFP for their bioluminescence, and hence produce blue rather than green light.

1.3.2 Bioluminescent Cnidaria

Many examples of imidazolopyrazine bioluminescence occur in the phyla of Cnidaria and Ctenphora. Bioluminescent species exist in all the three classes of the Cnidarians. This phylum was previously known as coelenterata. The three classes of the cnidaria are anthozoa, hydrozoa and scyphozoa (Shimomura & Flood, 1998).

All members of the cnidarian phylum are radially symmetrical animals with a two layered body i.e. an ectoderm and endoderm. Most have a lifecycle with two phases. One phase is a sessile polyp and the second phase is a free floating medusoid stage (Hayward & Ryland, 1994). Luminous species are present in all the three main classes of Cnidarians i.e. Hydrozoans, Anthozoans and Schyphozoans. All the luminous hydrozoans so far discovered have a photoprotein system of light production where the luciferin is coelenterazine e.g. obelin in *Obelia geniculata* and clytin in *Clytia hemisphaerica.* Bioluminescent hydrozoans are all thought to posses GFP in their photocytes and therefore their bioluminescent flash is green. In luminous anthozoa the bioluminescence is produced by a luciferin and luciferase reaction. The luciferin in this reaction is coelenterazine and most bioluminescent anthrozoa contain GFP. In contrast bioluminescent Schyphozoans have no GFP and some utilise photoproteins where others have classic luciferin and luciferase biochemistry (Campbell, 1988; Shimomura, 2006).

1.3.2.1 Bioluminescent Hydrozoans

Hydroids have a complex life cycle involving both a polyp and free swimming medusa stage. The existence of a velum inside the medusa bell and the production of gametes from ectodermal tissue are two of the features that distinguish them from other Cnidarians (Cornelius, 1995). In the hydrozoa the sessile polyp stage of the Cnidarian life cycle predominates. Some hydroid polyps are specialised for specific functions e.g. hydranths for feeding and gonothecae for reproduction. This diverse subgroup includes *Obelia geniculata, Velella velella* and *Physalia physalia* more commonly known as the Portuguese man-o-wars (Hayward & Ryland, 1994). Other marine organisms can be observed living on and in colonies of *Obelia.* Nudibranchs live on colonies of *Obelia* eating the hydroids. Amphipods can also cohabit with *Obelia* (Bavestrello et al. 2008). During this research project amphipods have been observed living in cocoons they have made on Pembrokeshire specimens of *Obelia longissima (*refer to Fig 1.7*). Obelia* is an epiphyte in that algae live symbiotically within its stem. Algal growth on and in the hydroids increases as the colony ages.(Bavestrello et al. 2008). Many samples of *Obelia geniculata* taken from Martin's Haven in Pembrokeshire appeared pink due to the red algae growth within them.The structure and life cycle of *Obelia geniculata* is discussed in chapter three.

Fig.1.6. A specimen of *Obelia longissima*. Image taken in late spring under high power, showing the feeding tentacles which retract if it is removed from sea water for any length of time.
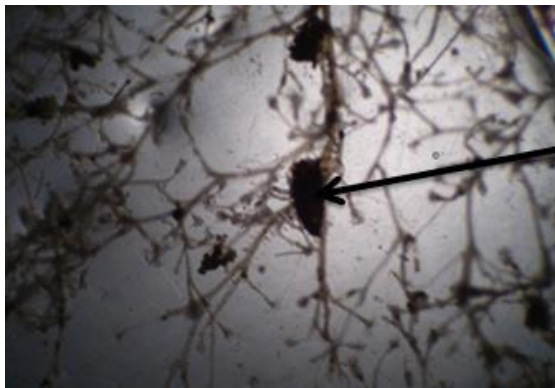
Fig.1.7. Amphipod cocoons. Amphipods live in cocoons within a colony of *Obelia longissima.* Image taken of a specimen from Milford Haven marina.

For this thesis I am studying bioluminescence in hydrozoans. All the biochemical studies on bioluminescence in this class of organisms to date, have established that photoproteins activated by calcium ions are responsible for the light emission they produce. In addition the luciferin component of the photoprotein is always coelenterazine and GFP is also present. The GFP lengthens the wavelength of light produced (Shimomura & Flood, 1998). Within the order of Hydroida there are many examples of organisms that exhibit bioluminescence (refer to Table.1.2.). From the time of Claudius Aelianus (a Roman author) onwards "many naturalists have noted the sparkling when the hand is rubbed over hydroids growing on rocks

21

or piles in the sea at night". Aelianus's work is a source of medieval natural history and includes many references to marine organisms (Harvey, 1952).

Order	Family	Genus
Hydroida	Aequoridae	*Aequorea*
	Campanulariidae	*Campanularia, Clytia (polyp), Phialidium (medusa), Obelia*
	Lovenellidae	*Lovenella*
	Mitrocomidae	*Halistaura (Mitrocoma)*
	Paneidae	*Leuckartiara, Stomotoca*
	Phialuciidae	*Octophialucium*
	Tubulariidae	*Euphysa*
Siphonophora	Abylidae	*Abyla, Abylopsis, Bassia, Ceratocymba*
	Agalmidae	*Agalma, Halistemma, Nanomia*
	Apolemidae	*Apolemia*
	Diphydae	*Chelophyes, Diphyes, Sulculeoaria*
	Forskaliidae	*Forskalia*
	Hippopodiidae	*Hippopodus, Vogtia*
	Prayidae	*Amphicaryon, Maresearsia, Nectopryramis, Praya, Rosacea*
	Rhizophysidae	*Rhizophysa*

Table.1.2. Examples of luminous hydrozoans. Adapted from Herring 1987.

Charles Stewart made one of the earliest studies of luminous hydroids. His book "Elements of the natural history of the animal kingdom" (published in 1802) includes his account of *Sertularia pumila* on *Fucus.*

"This species in some particular states of the atmosphere, gives out a phosphoric light in the dark. If *Sertularia* receives a smart stroke with a stick in the dark the whole coralline is most beautifully illuminated with every denticle seemingly on fire".

Paolo Panceri (1833 to1877) identified luminous cells and studied stimulation of hydroids. Harvey describes him as the greatest Italian student of bioluminescence. His papers discuss many examples of marine bioluminescence including pennatulids, ctenophores, siphonophores and hydroids. In 1841 M. Sars first deduced the alternation of the generations for hydroid and medusa (Harvey, 1957). "There are many species of *Aequorea* and *Obelia*, and possibly all of them are bioluminescent" (Shimomura, 2006). Other hydroida that are known to be luminous include, *Mitrocoma*, *Clytia and Phialidium.* Whilst aboard HMS Beagle (1831 to 1836) Charles Darwin recorded observing luminescence in a *Clytia* like zoophyte whilst collecting specimens at Tierra del Fuego (Harvey, 1952). This may have been luminous medusa of *Clytia hemisphaerica.*

All species of *Obelia* produce green bioluminescence due to the presence of GFP; this poses the question as to why it is advantageous for these marine organisms to emit green rather than blue light. This is a key question the answer to which is still being sought. For a further discussion of the role of GFP in the different species of *Obelia* and hypothesis for its evolution refer to chapter six. The photocytes of

Obelia contain the photoprotein obelin. This is activated by calcium ions and can be extracted from the hydroid *Obelia geniculata* (Campbell, 1974). Under the fluorescent microscope the position of *Obelia geniculata's* photocytes can be clearly seen as they contain GFP. Hastings and Morin defined photocytes as cells specialised for light emission and were the first to establish that *Obelia geniculata's* photocytes all contain GFP (Hastings, 1996; Hastings & Morin, 1998). Obelin the photoprotein in *Obelia* reacts in a very similar way to aequorin and its regeneration from apoobelin and coelenterazine can be utilised to assay for coelenterazine levels in marine organisms (Campbell & Herring, 1990). Morin and Cook measured the bioluminescence of *Obelia* by stimulating it with potassium ions. This depolarises the membrane of the hydroid's excitable epithelial cells and results in flashes of light scintillating along the colony (Morin & Cooke, 1971a).

In the 1980's Dunlap et al and Brehm et al concluded that prior to luminescence calcium ions enter non-luminous support cells adjacent to the photocytes. They proposed that calcium ions then diffused into the photocytes through gap junctions. Once calcium ions enter the photocyte they bind to the obelin producing a rapid flash. However this model doesn't fully explain the wave like spread of light that is observed in *Obelia* when new areas of scintillating luminescence appear. It may be that there are undiscovered calcium ion channels in the plasma membrane of the photocytes (Dunlap et al. 1987). The different theories for how calcium ions enter the photocytes are discussed in chapter 4.

When calcium ions trigger obelin inside the cells of *Obelia geniculata* an incredibly rapid flash of bioluminescence is produced with 300ms kinetics. If this reaction is carried out *in vitro* the reaction is slightly slower and has a rate constant

of only $4s^{-1}$. This difference in kinetics between the *in vivo* and *in vitro* reaction has never been fully explained and is a key question which remains to be answered (Campbell, 1994).

1.3.2.2 Bioluminescent Anthozoans

So far bioluminescence in this class of marine organisms has been found to be produced from luciferin-luciferase type reactions rather than by photoproteins. The luciferin in all these reactions is coelenterazine. Most bioluminescent Anthozoans contain GFP (Shimomura & Flood, 1998). Members of this class of Cnidarians completely lack the medusoid stage of their life cycle and only the sessile polyp is seen (Hayward & Ryland, 1994).

Renilla reniformis is an example of a bioluminescent anthozoan which has been the subject of a great deal of research. *Renilla reniformis* is commonly referred to as the sea pansy. Ellis in 1763 was almost certainly referring to *Renilla reniformis* when he recorded a luminescent specimen of a kidney shaped purple sea pen in South Carolina. Harvey in his book on bioluminescence discusses the early experiments of Parker in the1920's. Parker describes *Renilla* as "having many zooids, which make up a kidney shaped colony on a stalk". Parker identified two types of material in the sea pansy which he said were associated with what he termed its "phosphorescence". He recorded them as a whitish chalky material and a yellow crystalline material. On the outer fringe of *Renilla* he proposed that light emission was associated more with the whitish chalky material (Harvey, 1957). The first coelenterazine luciferin was isolated and partially characterised from this anthrozoan (Cormier, 1961). This was the results of the work of Dr Milton

Cormier's research group at the University of Georgia. A tiny sample of coelenterazine was isolated by Cormier from *Renilla* in the mid 1960's but at the time its chemical identity was unknown (Hori & Cormier, 1965). The discovery of coelenterazine is attributed to Shimomura in the 1970's because he identified its detailed structure (Shimomura, 1974; Shimomura, 1987). The work of two separate research groups (Inoue et al. 1975) and (Hori et al. 1977) contributed more information on coelenterazine in *Renilla*.

Renilla luciferase which catalyses the bioluminescent reaction in the sea pansy was isolated and characterised by the end of 1977 (Karkhanis & Cormier, 1971; Matthews et al. 1977). The molecular weight of the pure *Renilla* luciferase has been established to be 35,000. *Renilla* luciferase catalyses the luminescence of coelenterazine when oxygen is present. The optimum temperature for this reaction is 32 ^{0}C and pH7.4 (Shimomura, 2006). Cormier in his original work on the luciferin- luciferase reaction of *Renilla reniformis* in 1961 thought that the reaction needed ATP, ADP or AMP to proceed later he established that the exact co-factor required is 3' 5'-diphosphoadenosine. It is this molecule which activates the coelenterazine. Hori et al have found that this activation works by a hydrolysis reaction removing a sulphate group from the luciferin (Hori et al. 1972). For this reason the original luciferin is now called luciferyl sulphate and is a sulphated form of coelenterazine (Shimomura, 2006). *Cavernularia obesa* is a bioluminescent anthozoan commonly called the sea cactus, which also uses coelenterazine as a luciferin to produce light. It lies hidden in the sand during the day but emerges at night as it absorbs seawater causing it to expand. It is also reported to contain GFP (Shimomura & Johnson, 1975b). Harvey described the

bright luminous slime produced from *Cavernularia obesa* when it is stimulated by touch. He investigated whether temperature affected its bioluminescence. He found that it would still produce a bioluminescence response at 0^0C but that its bioluminescence was irreversibly destroyed at temperatures above 52^0C (Harvey, 1957). This is most probably due to heat denaturing its luciferase.

Fig.1.8. *Pennatula phosphorea.* A Specimen of *Pennatula phosphorea* the bioluminescent seapen. A kind gift from Prof A.K.Campbell.

Anthozoans include the order of Pennatulacea *A* typical example is *Pennatula phosphorea* a bioluminescent sea pen All members of the *Pennatula* order are thought to be capable of bioluminescence. The Romans knew of the existence of sea pens which they called sea feathers "Penna marina" but did not record any examples of their bioluminescence (Harvey, 1952). *Pennatula phosphorea* produces a bioluminescent display when disturbed in the dark (Shimomura, 2006). It is a deep red colour and has a thick axial polyp which acts as an anchor in the soft mud or sand of the sea pen's location.

1.3.2.3 Bioluminescent Scyphozoans

In this class the medusoid stage of the life cycle predominates and the polyp stage is only seen (if at all) in minute larvae form (Hayward & Ryland, 1994; Shimomura, 2006). The exumbrellar is the term used to describe the upper surface of the medusae. None of the bioluminescent scyphozoans that have been studied so far appear to contain GFP (Shimomura & Flood, 1998).

Periphylla periphylla is an example of a luminous scyphozoan. Dense populations of this medusa are particularly found in Norwegian fjords. Its bioluminescence is thought to be produced in two different anatomical parts of the medusa. It has photocytes in its exumbrellar epithelium, but also has cytoplasmic granules in its ovarian eggs. It is thought to produce light using a luciferin-luciferase system. The luciferin it uses is coelenterazine. Interestingly it appears to have two different luciferases. One luciferase is thought to catalyse the bioluminescence in its ovary and a second luciferase acts as the enzyme in the luminescent reaction of its photocytes. Studies have shown that once an egg of *Periphylla periphylla* is fertilised the amount of luciferase begins to decrease. Conversely "large adult specimens contain very large amounts of luciferase" (Shimomura, 2006). Exactly why the luciferase level in the eggs decreases is a complex problem, which still needs to be solved (Shimomura & Flood, 1998).

Pelagia noctiluca is another example of a luminous scyphozoan, but it does not contain luciferin luciferase biochemistry. Edmund N. Harvey observed that a luminous extract from this jellyfish will emit light even in the absence of oxygen. This was the first clue that *Pelagia noctiluca* did not possess a classic luciferin

luciferase bioluminescent system (Shimomura, 2006). Harvey observed that when stimulated by touch *Pelagia noctiluca* produced bioluminescence on its whole outer surface including its tentacles. He postulated its bioluminescence response spread across the scyphozoan via a nerve net (Harvey, 1957). It was not until the 1970's that Jim Morin reported a successful extraction of a photoprotein from *Pelagia noctiluca* that was triggered by calcium ions (Morin & Hastings, 1971a; Morin & Reynolds, 1972). This illustrated that within the scyphozoa some bioluminescent species utilise photoproteins whereas others use luciferin-luciferase reactions.

1.3.3 Bioluminescent Ctenophora

Commonly referred to as comb jellies these organisms are transparent with radially symmetrical gelatinous bodies. Each member of this marine phylum has eight series of swimming combs, which have cilia. Mainly found in the pelagic zone of the sea they use tentacles to capture surface plankton (Hayward et al. 1996). Many Ctenophora are bioluminescent and two species *Beroe ovata* and *Mnemiopsis* have been found to utilise photoproteins (Ward & Seliger, 1974). The emission of light is triggered by calcium ions but unlike the photoproteins in *Obelia geniculata* and other bioluminescent hydroids Ctenophora photoproteins are light sensitive. "These proteins are photosensitive and inactivated by exposure to visible light; thus they are distinctly different from the hydrozoan photoproteins" (Shimomura & Flood, 1998). The luciferin bound in a complex within the photoprotein has been identified as coelenterazine (Anctil & Shimomura, 1984). Bioluminescent Ctenophora do not contain GFP.
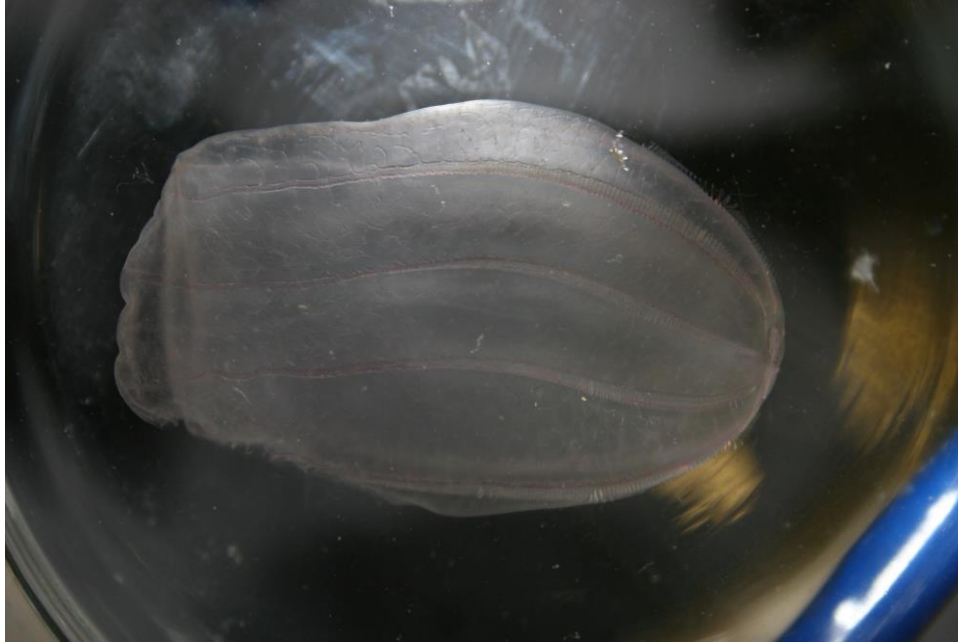
Fig.1.9. A specimen of *Beroe*. This organism is ctenophore which has no GFP and therefore produces blue bioluminescence. Specimen collected at Newgale Pembrokeshire, photographed with the binocular microscope under high power and using the Panosonic Lumix camera.

1.4 Applications of bioluminescence

Aequorin, obelin and GFP have been shown to have a wide range of applications in medicine and biotechnology (Campbell, 2003b). For example genetic engineering can now be used to insert the DNA coding for photoproteins into living cells. The light emitted enables free Ca^{2+} to be measured in parts of the live cell (Campbell et al. 1989; Freeman & Ridgway, 1991; Sala -Newby et al. 2000). "Because they are triggered by $Ca^{2+,}$ to produce light, photoproteins have been widely used as calcium reporters" (Haddock et al. 2001). In particular aequorin has been utilised to monitor calcium ion concentration in living cells. Ashley and Ridgway utilised aequorin "to follow the rapid changes in intercellular calcium concentration that occur during the contraction of single muscle fibre from the barnacle *Balanus nubilus*" (Ridgway & Ashley, 1967; Ashley, 1970; Shimomura,

2006). Ca^{2+} is a universal intracellular signal in all animal, plant and some microbial cells (Campbell, 1983). Calcium ions are essential in many in vivo biochemical reactions such as muscle contraction and insulin secretion (Campbell, 1983). Rainbow proteins have now been engineered which combine two bioluminescent proteins together. These will change colour when they react with a substance of biomedical interest (Waud et al. 2001). Hence rainbow proteins can be utilised in medical assays and give very high sensitivity. Their other advantage is that they can easily be used in assay kits where all test reagents need to be placed in a single tube. It is predicted that this technology will be used in future to test patients for a range of cancers as well as heart disease (Campbell, 2003b).

1.4.1 The functions of marine bioluminescence

"The evolution of bioluminescence signals has frequently occurred in environments where the ambient light is too dim to be an effective source of reflected light. The deep oceans comprise the most extensive and ancient of these, and it is here that bioluminescent signals are most widely employed" (Herring, 1990). Bioluminescence is used by marine organisms for defence, luring prey or communication e.g. attracting a mate (Campbell, 1994). One example of communication utilising bioluminescence is "schooling". This is when marine organisms use patterns of light to recognise members of their own species in the dark depths of the ocean. *Vargula (Cypridina)* produces its pattern of flashing dots by squirting its luciferin and luciferase into the surrounding seawater (Morin, 1986; Haddock, 2006). William Beebe famously observed the photophore patterns

that lantern fish display to recognise one another whilst descending in his bathysphere.

Many marine organisms including decapod shrimps and hatchet fish utilise bioluminescence from photophores for camouflage (Haddock, 2006). Decapod photophores are located on their cuticle and in hepatic tissues. The bioluminescence they produce exactly matches the light that comes down to them from the upper ocean. This counter illumination means they cast no shadow and are practically invisible to larger predators (Haddock, 2006). Bioluminescence can also be employed to scare away potential predators; an example of this can be seen in the brittle star *Amphipholas squamata*. This brittle star has photophores on all its arms but not its central disc. It has been seen to throw away a flashing arm as a sacrifice to confuse predators (Grober, 1988; Dewel & Mallefet, 2002; Haddock, 2006). Some decapods can also secrete a cloud of blue luminescence from their mouths. This is also thought to be a defence mechanism.

1.4.1.1 Bioluminescence and fluorescence in *Obelia*

Obelia geniculata is thought to use bioluminescence to deter predators and is stimulated by touch (Hastings &Morin, 1998).

There are several different species of the hydroid *Obelia* all of which are thought to be luminous. *Obelia geniculata* grows on seaweeds and is often found on *Laminaria*. Its medusa (one form of which was previously called *Obelia lucifera*) is also thought to be luminous. F. S. Russell described 3 species of *Obelia* hydroids in his medusae of the British Isles (Russell, 1953). He stated that two forms of *Obelia* medusae had been observed. *Obelia lucifera*, that was thought to

arise from *Obelia geniculata* and, *Obelia dichotoma*, and *Obelia nigre* that was presumed to arise from the hydroid *Obelia longissima.*

Russell was unsure as to whether any of the hydroids or medusa, were in fact separate species and stated that "the truth would only be reached by genetic research". He explains that *Obelia* medusae are commonly found in coastal plankton throughout the Britain, but describes their species identity as being "in a state of chaos".

In the 1990's authors were still referring to similar difficulties compounded by the fact that four species of Obelia were now recorded: - *Obelia geniculata, Obelia dichotoma, Obelia longissima and Obelia bidentata.* Ryland and Hayward state that there is a large amount of morphological variation within some species of Campanularidriidae, and that young colonies of hydroids may lack some characteristics of older colonies, making them difficult to identify (Hayward & Ryland, 1990). Paul Cornelius has produced a key to taxonomically distinguish the 4 species of hydroid, but also refers to problems in distinguishing one from another (Cornelius, 1995). The presence and pattern of GFP in *Obelia's* hydroids and medusae may be a valuable tool to clearly distinguish between these supposed different species. It could form a rapid taxonomic tool. However sequencing the DNA of all the different forms would be the only way to confirm which separate species are. Hastings and Morin observed different positions of photocytes in different species of *Obelia* describing them as "dispersed in an upright of *Obelia geniculata*" and "concentrated in the tip of a pedicle" in *Obelia dichotoma* (Hastings & Morin, 1998).

The difference in position of photocytes may have evolved due to *Obelia longissima* and, *Obelia dichotoma* deterring a specific predator from eating their hydranths. This predator may be a nudibranch. Different species of nudibranch are known to eat different parts of the hydroid. For marine biologists, observing this photocyte fluorescence, could aid identification of the different species of *Obelia.* It also rapidly distinguishes *Obelia* species from the non luminous *Laomedea flexuosa* which in the past has often been misidentified as *Obelia*. It is a straight forward procedure to grow most species of hydroids in seawater tanks. However culturing *Obelia* species can be very problematic. In the past scientists have cultured what they thought was *Obelia* only to discover that it was in fact *Laomedea (*Crowell, 1953*)*. Chapter three of this thesis explores whether bioluminescence and fluorescence can be utilised to accurately distinguish the four reported species of *Obelia*.

1.4.2 The evolution of bioluminescent proteins.

Organisms in the same phyla can have very similar luciferases. Anthony Campbell's research group have shown that the DNA sequence which codes for the luciferase producing green light in *Lampyris notiluca* (the glow-worm) is 80% identical to that which produces the yellow emitting luciferase of *Photinus pyralis* (the fire fly*)*. A difference of a few amino acids can result in a different coloured bioluminescence despite the fact that both these organisms use an identical luciferin (Sala-Newby et al. 1996).

E. N. Harvey working in the 1950's highlighted that the random occurrence of bioluminescence in some phyla but not others, made the evolutionary pathway for

34

bioluminescent systems difficult to predict. "It is as if the main groups of organisms are listed on a blackboard, and a handful of sand is thrown. The luminous species are where the sand sticks" (Harvey, 1952; Campbell, 2003). J. Woodland Hastings has predicted that bioluminescence must have evolved on 30 separate occasions and occurred after the evolution of vision (Hastings, 1983: McCapra, 1990). He has also hypothesised that luciferases originally evolved due to their ability to act as" oxygenase enzymes" for vital reactions (Hastings, 1983; Herring, 1987). More recently Campbell has formulated the solvent cage hypothesis which postulates that; "Bioluminescent proteins may have initially evolved as a solvent cage consisting of a few key amino acids" (Vassal et al. 2012). Chapter seven of this thesis explores the solvent cage hypothesis and the key question, "what is the evolutionary origin of coelenterazine?"

1.4.3 The hypothesis for coelenterazine synthesis.

Bioluminescence is utilised as a major communication system in the deep oceans. Coelenterazine is the luciferin responsible for the majority of bioluminescent reactions in the sea (Shimomura et al, 1980; Thomson, et al. 1995). Coelenterazine an organic luciferin was originally discovered by Osamu Shimomura (Shimomura, 1987). The overall objective of my research project is to understand the origin of bioluminescence in *Obelia*, to discover if *Obelia* relies on a dietary source of coelenterazine or has a biosynthetic pathway for the luciferin.

Coelenterazine is found in many other marine organisms apart from coelenterates. In fact it is the most common chemistry responsible for bioluminescence in the sea (Shimomura et al. 1980). It is responsible for bioluminescence in at least 8 marine phyla. Many non-luminous organisms have been shown to contain relatively large

amounts of coelenterazine. Several coelenterate bioluminescent genes have been cloned. How the marine organisms obtain the luciferin component of their bioluminescent reaction, is a matter of debate. There are two scientific theories on the source of coelenterazine. Either the luciferin is obtained through the diet, or luminous marine organisms are capable of *de novo* synthesis of the luciferin. If the answer to this key question could be discovered it would be of major importance in understanding some of the food chains in the sea especially those which involve Cnidarians. It may well be that the solution is that both processes are occurring. From current evidence different species may obtain their luciferin in different ways and there is unlikely to be one solution to this question. The evidence supporting both theories is reviewed in chapter five. A key question is how *Obelia* colonies in Pembrokeshire obtain their coelenterazine? This question is also explored in chapter five of this thesis.

More recently, in 2009 Oba and Kato have used deuterium labelled amino acids and electrospray ionisation-ion trap-mass spectrometry to show that the luminous copepod *Metridia pacifica* is able to synthesise coelenterazine. They have demonstrated that the copepod will synthesise coelenterazine from one molecule of L-phenylalanine and two molecules of L-tyrosine (Oba & Kato et al. 2009). Campbell and Herring have postulated that such luminous coelenterazine containing copepods, may act as a source of luciferin for other bioluminescent species (Campbell & Herring, 1990).

The hypothesis for coelenterazine synthesis is via a cyclisation of three amino acids FYY (phenylalanine, tyrosine, and tyrosine). It is proposed that the tripeptide undergoes dehydrogenation and decarboxylation before cyclising. This

is similar to the cyclisation of the chromophore in green fluorescent protein (GFP),

from SYG (Serine, tyrosine, glycine) and the yellow and red chromophores now

discovered in corals (Campbell, 1988; McCapra, 1990). Vargula luciferin is

already known to be synthesised from 3 amino acids argenine, isoleucine and

typtophan (Campbell, 1988; Kato et al. 2007).
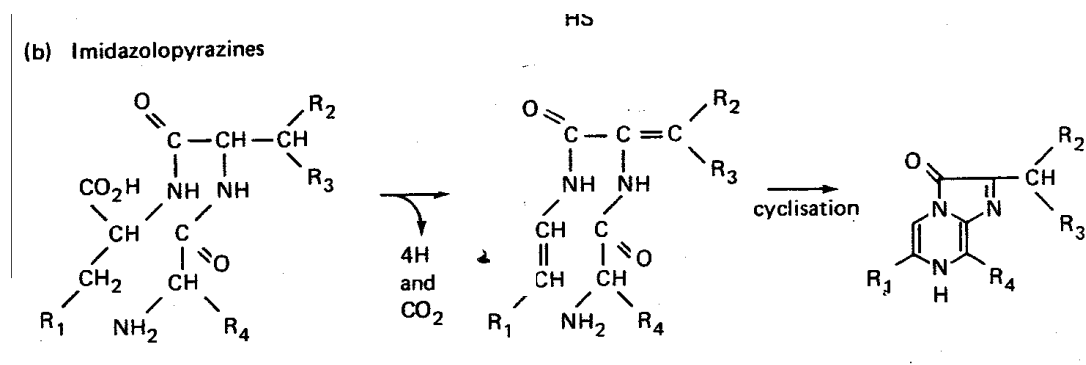
Fig.1.10. The biosynthesis of coelenterazine. The proposed mechanism for biosynthesis of coelenterazine is thought to occur via a cyclisation of 3 amino acids to produce the structure of coelenterazine. In the diagram R_1 and R_3 = phenol group, R_2 = H, and R_4 = CH_2 benzene ring (Adapted from Campbell, 1988).

Overall objective

To understand the origin and regulation of bioluminescence in *Obelia*.

1.5 Key problems

The key problems which this thesis is trying to address are:-

(i) The misidentification of *Obelia* species. It can be difficult to distinguish

between the four species of *Obelia*. In addition the luminous species

Obelia geniculata can be confused with the non-luminous *Laomedea*

flexuosa. The problem of identification was addressed in chapter three.

(ii) How is bioluminescence triggered in *Obelia*? Does each photocyte flicker independently, or is there a wave-like spread of bioluminescence down the colony? Stimulation of *Obelia* has been shown to produce a bioluminescent response from photocytes within milliseconds.The full details of how this response is produced are yet to be confirmed.This question was investigated in chapter four.

(iii)What is the origin of coelenterazine in *Obelia*? What precisely is the source of coelenterazine for colonies of *Obelia* in the Milford Haven waterway? Does *Obelia* obtain coelenterazine through its diet or is it capable of *de novo* synthesis of its luciferin? These questions were explored in chapter five.

(iv)Do all the Obelia species emit bioluminescence and fluorescence at the same wavelength? Does the wild type GFP present in *Obelia* photobleach? These questions were investigated in chapter six.

(v) What is the evolutionary origin of coelenterazine? Did coelenterazine evolve as a mono-oxygenase enzyme, and thus did it originally act as the marine equivalent of vitamin C? These questions were explored in chapter seven.

(vi) Can bioluminescence be utilised as an educational tool? Can it be used to teach a wide range of scientific skills and principles? These questions were investigated in chapter eight.

1.6 The strategy

(i) To investigate whether bioluminescence and fluorescence can be used for identification of different species of hydroid. To establish a method for using fluorescence and bioluminescence, to accurately identify specific species of hydroids and, to distinguish between, taxonomically similar bioluminescent and non bioluminescent species.

(ii) To investigate how bioluminescence is triggered through the supposed nerve-net in *Obelia*.

(iii) To identify potential sources of coelenterazine.

(iv) To develop albumin as a model, for the evolution of coelenterazine.

(v) To develop bioluminescence as an educational tool.

Chapter two

Materials and methods

Materials and methods

2.1 Materials

2.1.1 General reagents

2.1.1.1 Chemicals

Coelenterazine was supplied as a gift from Bruce Bryan (Prolume Inc). All other chemicals were obtained from Sigma-Aldrich Company Ltd (Poole, Dorset UK).

2.1.1.2 Solvents

Solvents were obtained from Sigma-Aldrich Company Ltd (Poole, Dorset UK). All solvents were AnalaR grade.

2.1.2 Composition of reagents and buffers

The following reagent and buffer compositions were used:

(a) Alkaline iodide: 15g potassium iodide, 50g potassium hydroxide, 1g sodium azide /100ml.

(b) Buffer L: five parts 1M sodium carbonate to one part 1M Sodium hydrogen carbonate pH 8.

(c) EDTA: a 1M stock solution was made up from Ethylenediamine tetraacetic acid disodium salt dihydrate pH 7.5 and stored at RT.

(d) KCl: a 0.5M stock solution was made up from Potassium Chloride pH 7.5 and stored at RT.

(e) 10% Manganese (II) chloride: 10g /100ml.

(f) Medium O:10mM Tris /pH7.5, 500mM NaCl, 1mM EDTA, 5mM mercaptoethanol, 0.1% gelatin.

(g) Luminol standard for chemiluminometer: In 200 µl total volume.

4 µl 50 µM luminol (in 0.2M KOH)

194 µl Buffer L

2 µl 10mM Cupric ammoniacal sulphate

100 µl of 0.1 vol hydrogen peroxide

The luminol standard placed in an LP3 tube gives a peak photon count of approx 11000 photons lasting > 3hrs.

(h) TE extraction buffer: 200mM Tris/HCl pH7.4, 10mM EDTA

(i) TE preinjection buffer: 200mM Tris/HCl pH7.4, 0.5mM EDTA

2.2 The Measurement of chemiluminescence

This was measured using either a chemiluminometer or a Photek molecular imaging system.

2.2.1 The chemiluminometer

Photons emitted from samples were measured using a chemiluminometer which had been constructed by the late Malcom Ryall. A chemiluminescent reaction produces a rise in light intensity which reaches a peak and then decays. The

reaction may occur rapidly as a flash or more slowly as a glow. The sensitivity and flexibility of a chemiluminometer depends on the specification of its five main parts discussed below (Campbell, 1988).

Fig.2.1. The chemiluminometer. The picture shows the photomultiplier tube.

(i) The sample housing must keep the sample in a dark environment, but also allow chemicals to be added to it to initiate the reaction. As much of the light as possible, produced in the sample must reach the light detector (Campbell, 1988). The sample tube is held in a rotating cylinder of brass. The sample housing is light–tight and the brass helps reflect the light produced in the LP3 tube towards the detector. It is important only to have a short distance between the sample tube and detector. Light emitted from the sample passes through a 3 x 420mm slit before reaching the detector. To initiate reactions a light tight injector is used. This consists of a 1ml plastic syringe and 20-gauge x 2.5 needle (Campbell, 1988).

(ii) A monochromator was not present on the chemiluminometer used as the complete spectrum of light being produced by the reaction was being measured.

(iii)The light detector is a photomultiplier tube (EMI 9747A) which has low current noise and high sensitivity (Thomson, 1995). Photomultipliers are very sensitive light detectors and provide a current output proportional to light intensity. Due to their large area light detection, high gain and ability to detect single electrons they have major advantages over other types of light detector (Campbell, 1988; Weeks, 1992). This photomultiplier tube is particularly sensitive to blue light and is fitted with a cosmic shield, which prevents random photons from the atmosphere reaching the tube.

Within the photomultiplier, light emission is detected at the photocathode. The photocathode emits electrons by the "photoelectric effect". The electrons are accelerated and focused onto the primary dynode within the multiplier tube. When they hit the dynode each electron frees a number of secondary electrons which are electrostatically accelerated and focused onto the next secondary dynode. This process is repeated through a chain of dynodes until electrons emitted from the final dynode are collected at the anode (Campbell, 1988). Another important feature of the tube is that it has stabilised high voltage, which prevents sensitivity varying over time.

(iv) The signal processor amplifies the output from the detector presenting it to the recorder. The original signal which hits the photomultiplier tube is in digital form i.e. photons. A digital chemiluminometer is used rather than analogue because it

has a more stable instrument noise, and is therefore more sensitive to low level chemiluminescence.

The signal processor has two parts consisting of a preamplifier and a discriminator. The preamplifier provides the discriminator with a pulse in an appropriate form. The discriminator minimises the background noise escaping from the digital processor. It does this by considering the energy level of pulses leaving the detector. Only pulses above a certain level are recorded. The level is selected to produce the optimum signal noise ratio. The discriminator must respond in nanoseconds otherwise pulses may be lost or distorted. Once the discriminator has converted the varying energy levels into uniform square wave pulses amplified 10 to 1000 times they are passed to the recorder.

(v) The recorder is an 8 digit scalar which displays the accumulated photon count (Campbell, 1988).

2.2.1.1 Optimisation of the chemiluminometer.

The effect of varying voltage on the signal to noise ratio of the chemiluminometer was investigated. This involved measuring the effect of varying voltage on the photon counts from a standard, and recording the effect of varying voltage on the machine blank.

The effect of varying voltage
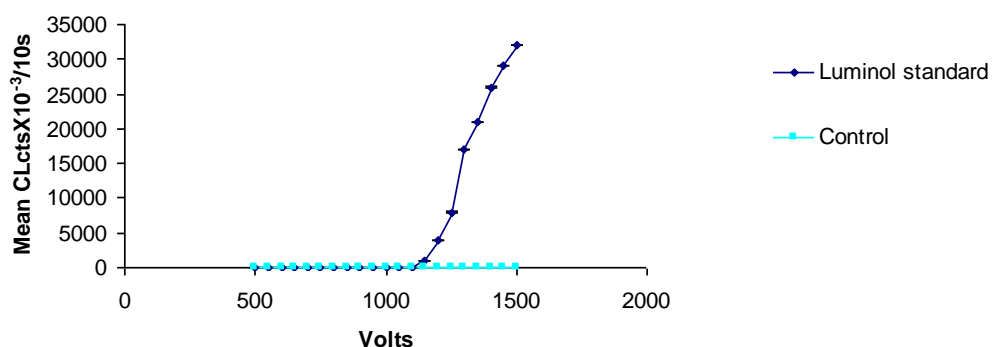
Fig.2.2. The effect of varying voltage. The mean photon counts from a luminol standard were measured in six replicates at different voltages. The lowest voltage setting of the chemiluminometer is 0 which represents a voltage of 500 volts. At 1250 Volts the photon counts rapidly rose with increasing voltage.

The signal to noise ratio
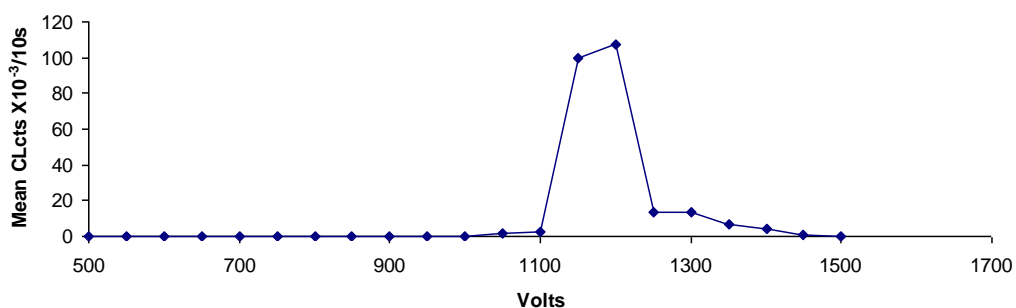
Fig.2.3. The signal to noise ratio. The mean from the luminol standard counts at each voltage setting was divided by the mean counts obtained from the machine blank (control), to give the signal to noise curve. This showed that 1200 volts gave the highest signal to noise ratio and that voltage was used for the chemiluminometer experiments. This corresponds to a setting of 7.0 on the chemiluminometer voltage control.

2.2.1.2 Measuring the bioluminescence of hydroids

To assess whether hydroids are luminous, potassium ions were used to stimulate the bioluminescent activity of individual hydroids (Morin & Cook, 1971). Photons emitted from the stimulated hydroids were measured by the chemiluminometer. A live hydroid was examined under the microscope to ensure its hydranths were intact; it was then carefully placed in an LP3 tube. After placing the tube in the luminometer and recording background readings, 1ml of 0.5M KCl was injected at five seconds to stimulate the hydroid to luminesce. Photon counts were recorded every second, over a two minute period. Bioluminescence of six hydroids was recorded to enable a mean bioluminescence to be calculated ± SEM.

2.2.2 The Photek imaging system

The imaging system was encased in a dark light tight box and the imaging camera was operated in a dark room at RT (refer to Fig 2.5). The Photek camera model 325 was used supplied by Photek Ltd, Hastings, Sussex, UK. The system uses an image intensified charge coupled device (ICCD), which produces an amplified electrical signal in response to photon emission. ICCD's are increasingly being used in biomedical research and astronomy. Examples of previous applications include the recording of calcium signals in plants (Campbell et al. 1996). Bioluminescence was triggered in live hydroid specimens by the addition of 0.5M KCl or by injecting 50mM calcium ions onto samples of photoprotein. The photons produced by the bioluminescent response hit the negatively charged photocathode, which is connected to a high voltage supply. This contains micro

channel plates (MCP's) derived from fibre optics .These produce an amplified electrical response and the resulting electrons are then accelerated towards the phosphor screen. This intensified signal is transmitted from the screen to the CCD camera which contains a silicon chip. The position of the CCD camera in relation to other key components in the system is represented in Fig 2.5. The CCD camera works at 50Hz which produces 50 frames per second. Each frame is 20milliseconds long. Every pixel on the silicon chip records a value of 1 or 0.Therefore in any given frame only one or two pixels will be positive. This lessens the memory needed and means that longer imaging run times can be used. Each pixel's value is recorded against its XY coordinates generating a SXY file. From this pixel data the image is reconstructed using the Photek software analysis system IFS216.
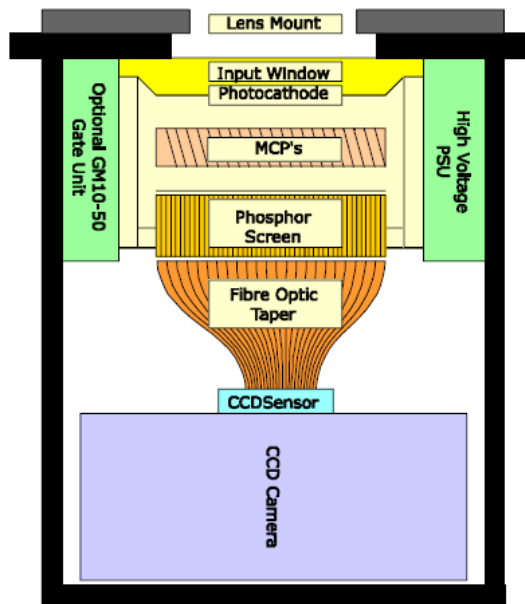
Fig.2.4. Diagram of the Photek imaging camera. This illustrates the position of key components including the micro channel plates and CCD camera. . http://www.Photek.com. Accessed 9.44 a.m. 13.02.12.
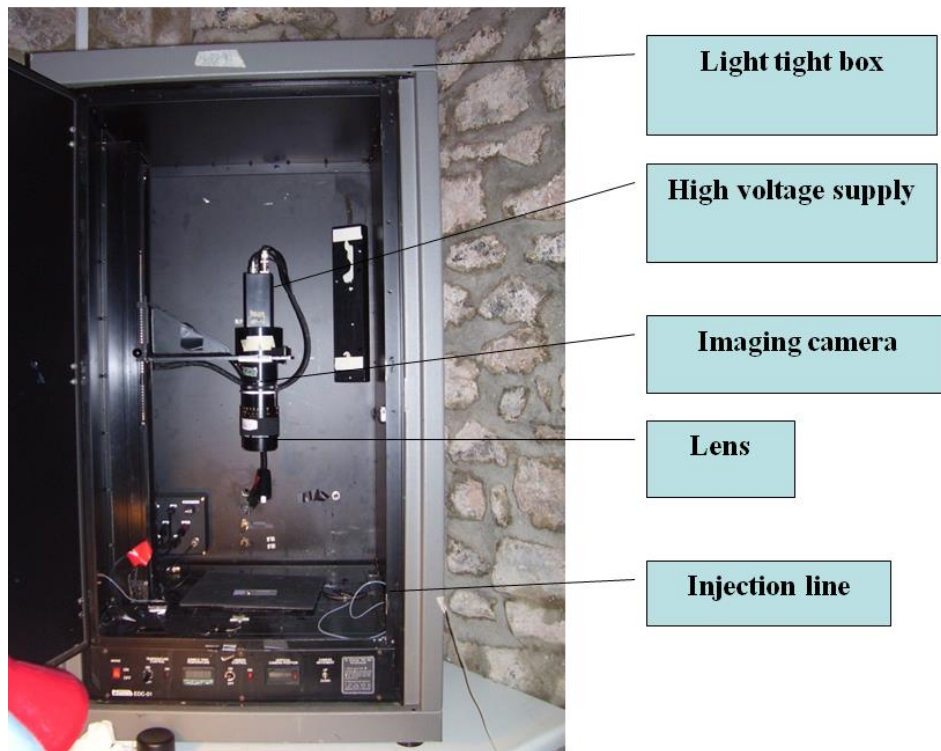
Fig.2.5. The Photek imaging camera. The picture shows the camera and injection line being set up in the light tight box.

2.2.2.1 Measuring spectra

To measure spectra from live specimens or photoproteins a diffraction grating and fibre optic were placed in front of the photocathode. The diffraction grating was set at between 450 to 550nm in order to capture blue green light.

2.2.2.2 The Photek colour palette

The Photek software allows a number of different colour palettes to be selected; the logarithmic colour option was used throughout. This utilises a pseudo colour palette to represent the photon emission intensity for each pixel of the total image. Red represents areas with high photon emission followed by yellow. The software assigns a colour to each pixel of the image depending on the level of photon intensity. The 8 bit intensity scale runs from 0 to188. Black represents zero photon

emission; blue represents a photon intensity of 1 to 2, whereas cyan represents an intensity of 3 to 7. Red represents areas with high photon emission i.e. 32 to 63 photons. Fig 2.6 illustrates this scale. This means that areas of high photon emission are easily discernible and resolution is far superior to a palette which only gives shades of white, grey and black.

2.2.2.3 Calculating odd area set data (OAS)

Photek analysis software was also utilised to measure the number of photons being emitted from each "hot area" i.e. red or magenta area. When imaging bioluminescence in hydroids these hot areas indicate the position of photocytes .and the pattern of that emission. Ten odd area set (OAS) circles are drawn on the brightest part of the total image from the specimen. Using the command extract OAS, the software then calculates the photons produced by each OAS (i.e. a hot area). An excel spread sheet was then used to graph the emission of each individual OAS area and show the pattern of photon emission for that photocyte. The same 10 circles were drawn on the black part of the image as well to extract the background emission which was subtracted from the results. OAS was also used in a similar way to assess the fluorescence of obelin on images taken during bleaching experiments, and the red, blue and green components of the fluorescence emitted from marine organisms.
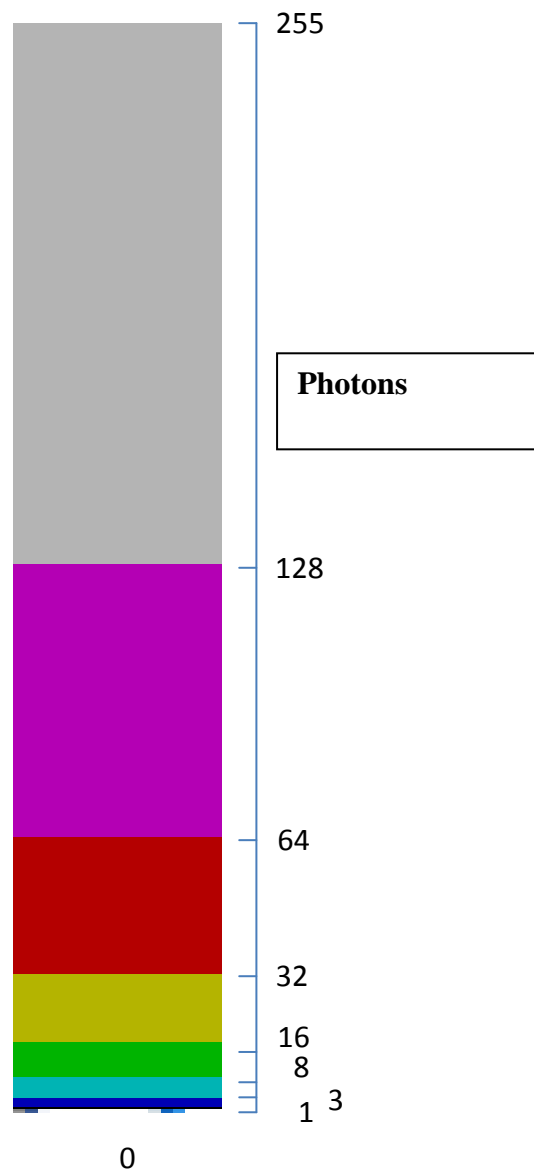
Fig.2.6. The Photek pseudo colour palette. An illustration of how photon intensity of each pixel is converted to a colour by the Photek software.

For example Fig.2.7 represents the total light emitted from the bioluminescent brittle star *Amphipholis squamata*. The logarithmic colour palette illustrates that light is emitted from photocytes in the brittle stars tentacles and not from its inner disc.
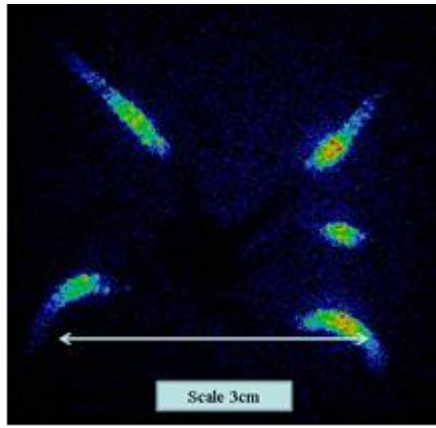
Fig.2.7. *Amphipholis squamata*.The total image acquired after *Amphipholis squamata* was stimulated with 0.5mls 0.5M KCl. Pseudo colour image taken with Photek imaging camera. The image was a kind gift from Professor Anthony K Campbell.
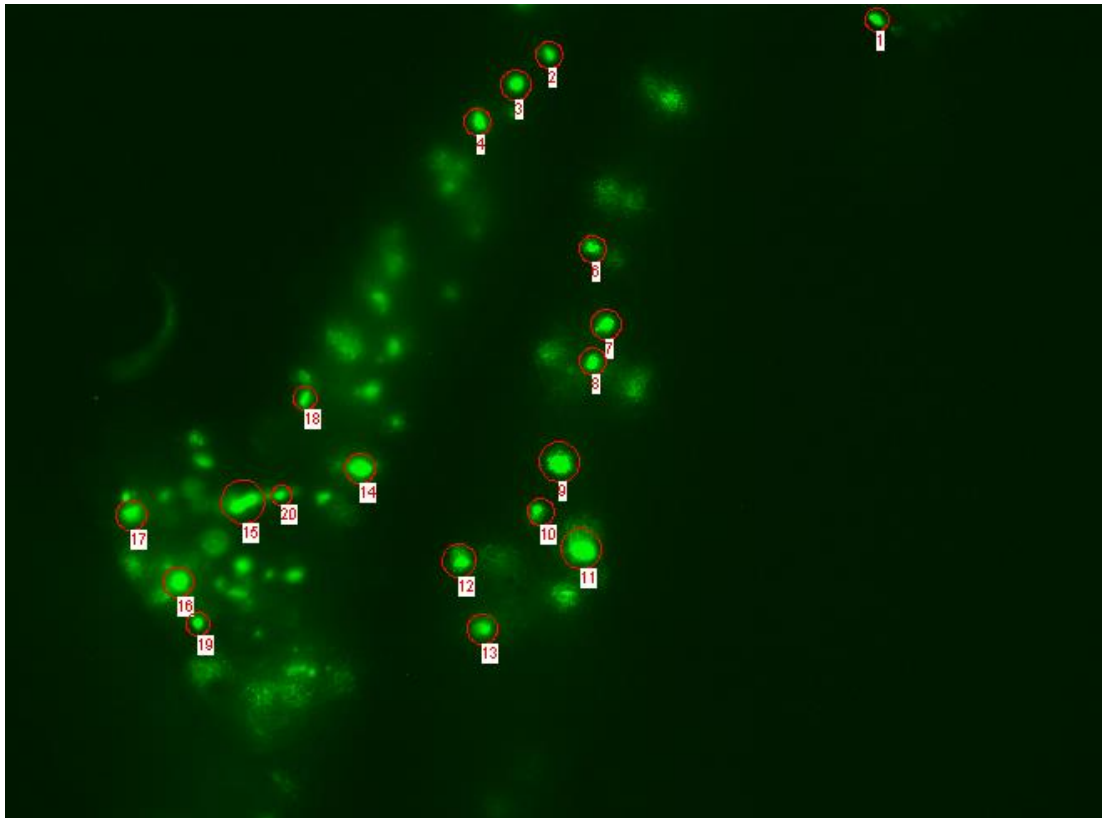
Fig.2.8. An example of odd area sets. Numbered areas of fluorescence were selected.The same set was used on non-fluorescent parts of the image to subtract the background.

2.2.3 Olympus bioluminescence imaging system.

The Olympus bioluminescence imaging system is known as the luminoview LV200. This relatively new system was designed primarily to study photosensitive cells over a long time course. It incorporates a Hamamatsu imaging camera which is an electron multiplication charged coupled device. It is particularly useful for studying live cultured cells as temperature, humidity, oxygen levels and carbon dioxide levels within the sample housing can be controlled. The system was tested to ascertain if it was suitable to record avi files of bioluminescent responses from individual hydroids, and whether it would simultaneously record bioluminescence and fluorescence in *Obelia's* photocytes. The camera and objective focus on the specimen from underneath the stage. The specimen was contained in a small glass Petri dish placed on the stage. A plastic injector line was taped to the side of the Petri dish.0.5mls of 0.5M KCl was injected onto the hydroid to stimulate its bioluminescence. Focusing on the specimen from beneath the stage proved problematical. In addition the base plate had to be altered in order for the Petri dish to lie flat. Bright field images of the hydroid were obtained, but they were not very sharp. The system has a large numerical aperture lens, which is difficult to focus on bright field. Sticking the hydroid onto the dish so it didn't move during the sequence was very difficult. Double sided sticky tape was tried, but this is auto fluorescent. Super glue and clear nail varnish were also experimented with. Super glue gave the best results, but there was still some movement during the injection so simultaneous recording of bioluminescence and fluorescence was not possible. Some audio visual interleave files (AVI) were obtained from *Obelia longissima* which show light emission, but the quality of these was not as good as those

obtained using the Photek imaging system. AVI files can contain both audio and visual data. They allowed a moving image of the bioluminescence to be recorded. The analysis system of the luminoview is less sophisticated than that of the Photek system. However the luminoview LV200 system does allow you to move very rapidly between bright field and fluorescent images. It is very quick and easy to overlay these images. Therefore the system is very useful for recording GFP patterns in hydroid photocytes over a period of time i.e. if hydroids or medusae are being kept in culture (refer to Figs.2.10. to 2.12.).

2.2.4 The extraction of obelin

Hydroids were wiped dry then five were placed in a 3ml glass homogeniser containing 500ul of TE extraction buffer. The homogenate was spun at 12,000g for 5mins. The resulting supernatant contains the photoprotein obelin. The photoprotein can be kept active on ice for a few hours or stored for several months frozen at -20^0C (Campbell, 1974; Thompson, 1995).

2.2.5 Measuring the activity of obelin.

Photoprotein was extracted using the above method. The activity of 3 aliquots of 50 ul of obelin extract was then measured. The extract was placed in 500 ul of preinjection TE buffer in an LP3 tube. The tube was placed in the luminometer and obelin activity stimulated by addition of 500 ul of 0.5 M calcium chloride. The photon emission was then recorded.

Fig.2.9.Images of *Obelia longissima* obtained from the Olympus luminoview
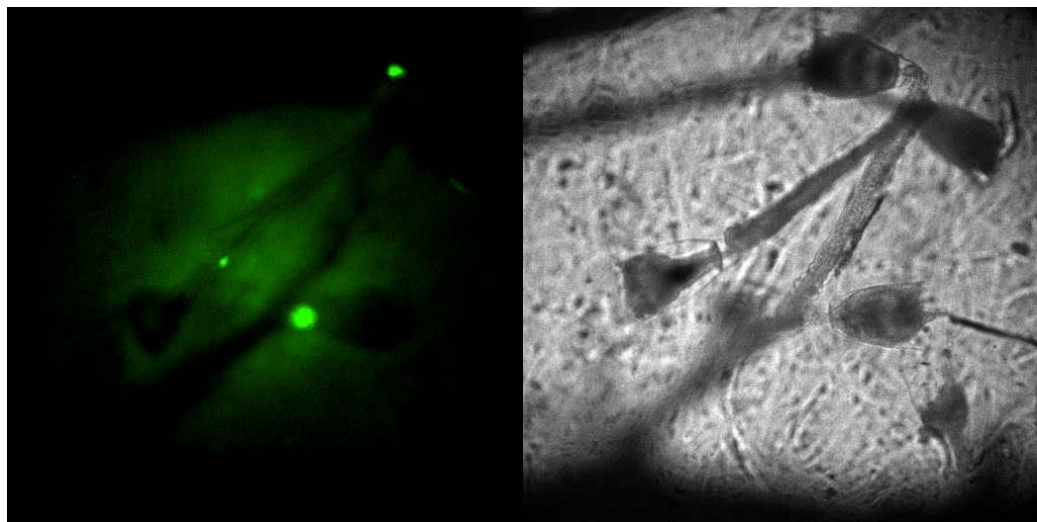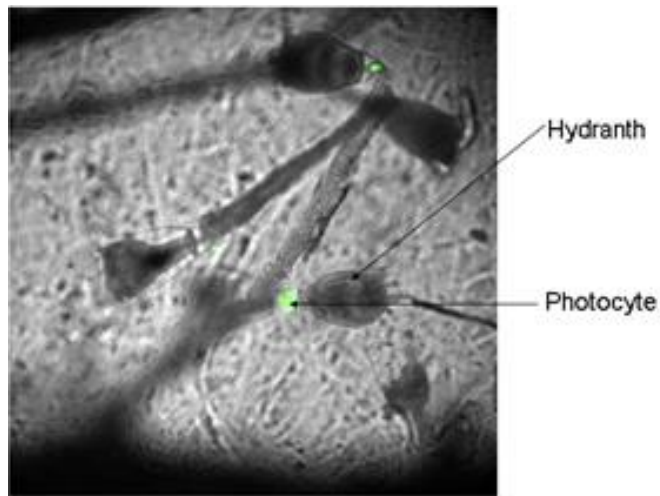
Images of *Obelia longissima* obtained from the Olympus luminoview LV200.
The top image shows the fluorescent and bright field image combined. The left image shows the fluorescence and the corresponding bright field image is on the right. All images were taken using a magnification of X 200.

2.3 Measuring and photographing fluorescence

 To observe the fluorescent photocytes of hydroids and medusae, blue light must be shone onto the specimen, so that the GFP absorbs it, producing electronically excited states. As these electrons move rapidly back down to a lower energy state the fluorescence can be observed. A Hund H500 fluorescent microscope was used fitted with fluorescein filters. The fluorescence was observed using the technique of epifluorescence. A mercury lamp was used as a light source. This light passes through the narrow band excitation filter (470nm to 485nm).The resultant blue light is reflected by a mirror down onto the specimen. Before reaching the specimen the light passes through a dichroic filter which only allows blue light to pass through. The blue light excites the GFP in the specimen. Only green light emitted by the specimen can now return through the dichroic and pass through the emission filter (510nm to 520nm). Green fluorescence is then observed at the eyepieces of the microscope. Fluorescence was photographed using either a Sentech camera or Nikon Coolpix 400. The filters for the microscope set 41020 were obtained from Chroma technology corp, 10 Imtec lane, Bellows falls, VT 0501 USA.

2.3.1 Fluorescence Lifetime Imaging Microscopy FLIM

As a fluor, GFP absorbs photons very rapidly to form an excited state. The decay of this excited state is far less rapid. The decay of the excited state causes it to emit green light. The half-life of this decay is measured and can be compared to standards e.g. there is a standard measurement for the decay of GFP. Other standards used for comparison are the fluors fluorescein and rhodamine.

Fluor	Standard FLIM measurement nanoseconds
Fluorescein (dianion)	4.1 ± 0.1
Rhodamine B	1.74 ± 0.2

Table.2.1. An example of FLIM Standards. The FLIM measurements for two common fluors. Adapted from http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1855982. Accessed 04.08.12. 7.30am.

This technique was used to establish that fluorescence observed in the hydranths of *Obelia dichotoma* was due to GFP. The FLIM machine used was from La Vision biotech (http://www.lavisionbiotec.com) and had a X16 detector with time correlated single photon counting. The machine used time domain FLIM. A microscope is integrated with the machine. GFP was excited by light at a wavelength of 525nm and the FLIM then produces a pseudo colour image which indicates the fluorescent lifetime. A laser and "nanosecond-level shutter" are utilised to measure the lifetime, which for any fluor will lie between 1 and 20 nanoseconds (http://www.olympusfluoview.com/applications/flimintro.html accessed 5.15am 07.11.12).

2.4 Measuring water quality

2.4.1 Measure oxygen levels.

These were measured in sea water and river water using the lovibond comparator and test kits, which were obtained from: Tintometer Ltd, Lovibond house, Solar way, Solstice park, Amesbury, SP4 7SZ, Tel 01980664800. A 100 ml glass bottle was completely filled with the seawater or river water sample. One ml 10% manganese (II) chloride was added to the bottle, followed by 1 ml alkaline iodide.

The bottle was sealed without any air bubbles and mixed thoroughly. It was allowed to stand for 5 minutes for the precipitate to settle. One ml concentrated sulphuric acid was then added and the bottle carefully shaken to mix. The sample cleared. Ten ml of untreated sample water was then placed into a 13.5 mm cuvette. The cuvette was place in the left hand side of the Lovibond comparator. Ten ml of treated seawater sample was then placed into another 13.5 mm cuvette and placed in the right hand side of the comparator. The 3/3 oxygen disc was placed in the comparator and viewed against north daylight. The disc was rotated until the colours match and the concentration was then read in the bottom right hand window. The result in the bottom right hand window is given in mg oxygen per litre water.

2.4.2 Measuring nitrate levels.

These were also measured in sea water and river water using the lovibond comparator and test kits. A plastic bottle was filled to the mark with 20 ml of water sample. One level spoonful of nitrate test powder and one nitrate test tablet was added to the water sample. The cap was replaced and the tube shaken for one minute and then allowed to stand for one minute. The sample was then inverted three times and then left to settle for a further two minutes. A 10 ml sample of untreated water was placed into a cuvette, and this was placed in the left hand side of the comparator, as a control. Ten ml of treated sample was placed into another cuvette and one nitrite LR tablet added, crushed and mixed to dissolve the sample. The cuvette was left for ten minutes and then placed in the right hand side of the comparator. Nitrate disc 3/124 was then inserted and the sample viewed against

white light. The disc was rotated until the colours matched. The concentration of the sample is then given in the bottom right hand window. Results are given as mg nitrate nitrogen per litre water. To convert this to mg. nitrate per litre water multiply the result by 4.4.

2.4.3 Measuring salinity

This was measured using a salt refractometer from RS South Wales, unit D8.2, Forest court, Treforest Industrial estate Pontypridd CF375UR. Each time salinity was measured, the mean of three readings was taken.

The instrument had an error of +/ - 0.2%.

2.5 Preservation of hydroid specimens

Specimens were immersed in 8% formaldehyde for 24hrs and then stored in 70% ethanol, 1% glycerol.

2.6 Measuring bioluminescence in a culture of *Obelia*

Six hydroid uprights were removed from culture plates every day. The bioluminescent response of each hydroid was measured using the chemiluminometer; full details of this method are in Section. 2.2.1.2.

2.6.1 Measuring obelin activity in culture

Each day 5 hydroid uprights were removed from the culture plates and photoprotein was extracted from them using the following method; 5 hydroids were wiped dry then placed in a 3 ml glass homogeniser containing 500 µl of TE extraction buffer (200 mM Tris/HCl pH7.4 10 mM EDTA). The homogenate was

spun at 12,000 g for 5mins. The resulting supernatant contains the photoprotein obelin. The activity of the extracted photoprotein was measured by the method stated in Section 2.2.5.

2.6.2 Measuring apoobelin levels.

Obelin was extracted from *Obelia* hydroids and details of this method have already been given in Chapter 2. In order to detect apoobelin it was first converted to the photoprotein obelin by incubating it with coelenterazine (these assay tubes were labelled set 1). It was vital to set up controls which just measured the original amount of obelin in the extraction (these assay tubes were labelled set 2). The activity of apo-obelin is then calculated by subtracting the activity of the extracted obelin from the total of the reformed and extracted obelin i.e. set1 minus set 2. Set 1 tubes contained 5 μl of obelin extraction, with 2.5 μM coelenterazine in 50 μl of buffer "O". These tubes were incubated in the dark for 2hrs. Set 2 tubes contained 5 μl of extracted obelin with 50 μl of buffer "O". These tubes were also incubated in the dark for 2 hrs. Triplicate tubes were used in order that mean results could be calculated.

2.7.1 Producing apoaequorin for the coelenterazine assay

One transformed bacterial colony was inoculated into 20 ml of LB containing 100 μg/ml carbenicillin. The transformed bacteria were grown over night at 37°C and were agitated on a shaker. The next day a further 300 ml of LB were added to the culture to give a total volume of 500 ml. Growth was allowed to continue until the optical density at 600 nm was between 0.3 and 0.6 IPTG was then added to give a final concentration of 1mM in the culture. The bacteria were then grown for a further 2 hrs at 37^{0}C. The culture was then centrifuged at 3000 rpm for

10mins.The supernatant was removed and resuspended in 10 ml of aequorin resuspension buffer pH 7.4.

Aequorin resuspension buffer.

Tris-HCl   20 mM

Na$_2$EDTA   1 mM

DTT   5 mM

The resuspended pellet was then sonicated, on ice for 15 seconds. The sonication was repeated five times, but the sample was allowed to cool down in between each sonication. Following sonication the sample was centrifuged at 3000 g for 30mins at 4 ^{0}C. The supernatant which contains the apoaequorin was then removed. The quality of the apo protein was then assessed by incubating an aliquot of the apo aequorin with coelenterazine to reform aequorin.

Reforming aequorin.

Add 10 nmol of coelenterazine to 2 ml of buffer "O". Take a 300 μl aliquot of this solution and mix it with 300 μl of the supernatant which contains apoaequorin. Incubate this mixture at RT for 1 hr to allow the photoprotein aequorin to reform. 50 μl of the incubated mixture was then added to 200 μl of aequorin assay buffer. The aequorin activity was then tested by activating the sample in the chemiluminometer by injecting 200 μl of 50 mM CaCl$_2$.

Aequorin assay buffer pH 7.4

Tris-HCl 200 mM

Na$_2$EDTA 0.5 mM

2.7.2 The coelenterazine assay

Stock coelenterazine was stored as 10 nmol aliquots in HPLC methanol dried under argon gas. The protocol for the coelenterazine assay is adapted from the methods used by Campbell and Herring (Campbell & Herring, 1990). The assay is based on the fact that under the correct conditions coelenterazine will reactivate apoaequorin or apoobelin, reforming aequorin or obelin. The activity of the reformed photoprotein can then be measured using the chemiluminometer (refer to Section 2.2.5). A coelenterazine standard curve was run each time that the coelenterazine assay was performed. The curve was produced from a set of coelenterazine standards which were prepared by diluting the stock coelenterazine with methanol (10^{-15} to 10^{-9} mol). Light will automatically oxidise the coelenterazine so, once prepared, the dilutions were rapidly incubated with apoaequorin and "medium O" (10 mM Tris, 0.5 M NaCl, 1 mM EDTA, 5 mM mercaptoethanol, 0.1% gelatin, pH 7.5) in the dark, at room temperature for 4 hrs. Each coelenterazine dilution was assayed in triplicate. Following incubation, 50 μl of each standard dilution was removed and placed in 500 μl of aequorin assay buffer, in an LP3 tube. The tube was placed in the luminometer and the activity of any reformed obelin stimulated by addition of 500 μl of 0.5 M calcium chloride. The photon emission was then recorded using a 10 second count interval.
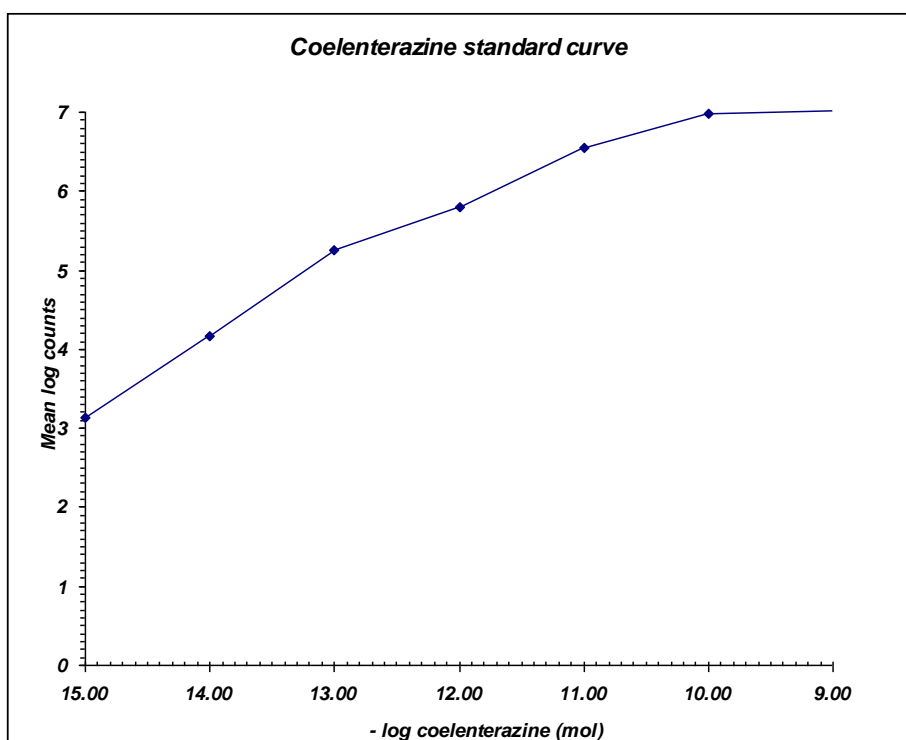
Fig.2.10. A standard coelenterazine curve, logarithmic plot. Apoaequorin (produced as described in 5.2.6) was incubated with dilutions of coelenterazine to form photoprotein. Photoprotein activity was triggered by addition of Ca^{2+}. Chemiluminometer counts recorded every 10s. Data points are the means of 3 observations $\pm$ SEM.

2.7.2. Extraction of coelenterazine from *Obelia*.

Fresh hydroids were briefly rinsed in 200 mM Tris/HCl, pH 7.4,10 mM EDTA, to remove seawater and then gently blotted dry on filter paper. Hydroids were homogenised on ice in 500 μl HPLC methanol, using a 3 ml glass homogeniser. Debris was removed by centrifugation at 12,000 g for 2 minutes in a microfuge. The supernatant was then kept at $4^{0}C$ using ice, and protected from the light. Extracts were then immediately tested using the coelenterazine assay. One μl of each coelenterazine extract was incubated with 5 μl apoaequorin and 95 μl medium "O" (10 mM Tris, 0.5 M NaCl, 1 mM EDTA, 5 mM mercaptoethanol, 0.1% gelatin, pH 7.5) in the dark at room temperature for 4 hrs. A portion of the

extract was placed in 500 µl of preinjection TE buffer, 200 mM Tris, pH 7.4, 0.5mM EDTA in an LP3 tube. The tube was placed in the luminometer and the activity of any reformed obelin stimulated by addition of 500 µl of 0.5 M calcium chloride. The photon emission was then recorded.

Chapter three

Identification and distribution of *Obelia*

3.1 Introduction

This thesis is focused on the bioluminescent hydroid *Obelia,* in particular the three *Obelia* species found in the Milford Haven waterway in Pembrokeshire i.e. *Obelia geniculata, Obelia dichotoma* and *Obelia longissima.* This chapter includes an investigation into whether bioluminescence and fluorescence can be utilised to accurately distinguish amongst these three species, which are often confused. *Obelia* is found below the tide line of the entire British coast and is practically cosmopolitan occurring in coastal waters throughout the world. Location of habitat was also investigated to determine if this is also an important aid to identification. Popular marine identification texts can be confusing regarding expected habitats for *Obelia geniculata.* The Collins pocket guide to the sea shore suggests *Obelia geniculata* is an intertidal organism as well as occurring sub littoral. An intertidal organism exists in the seashore area which is uncovered at low tide but covered in seawater at high tide. The term sub littoral means the zone continually covered in sea water, it extends from below the low tide mark to the boundary of the continental shelf. In fact *Obelia geniculata* colonies are always in the sub littoral zone.

3.1.1 Historical review and species indentification

Historically hydrozoan experts have given the hydroids and medusae of the bioluminescent *Obelia* species many different names. There has also been disagreement as to how many different species there are. Currently four different species of *Obelia* hydroid are thought to exist, but this does not match with the accepted number of medusae species which is only three. As the medusae arise

from the hydroid's life cycle, discussed in section 3.1.4, there must be four species of medusae. A further source of confusion originates from the fact that there are structurally similar non luminescent species, which are sometimes incorrectly identified as *Obelia*. *Laemodea flexulosa* (previously known as *Campanulariidae flexulosa)* is a non luminous hydroid which in the past has often been misidentified as *Obelia*. It is a straight forward procedure to grow most species of hydroids in seawater tanks. However culturing *Obelia* species can be very problematic. Historically some scientists have cultured what they thought was *Obelia* only to discover that it was in fact *Laemodea* (Crowell, 1953).

It can also be difficult to distinguish one *Obelia* species from another, particularly *Obelia dichotoma* and *Obelia geniculata*. This chapter investigates whether patterns of fluorescence and measurement of bioluminescence can form a rapid taxonomic tool to ensure correct identification of species. It is essential that scientists studying the species of *Obelia* can be confident that they have obtained the correct live specimens.

F.S. Russell produced in the 1950's what is still considered today to be an authoritative work on hydroids and medusae of the British Isles. He was unsure as to whether any of the hydroids or medusae of *Obelia* were in fact separate species and stated that "the truth would only be reached by genetic research". He explains that *Obelia* medusae are commonly found in coastal plankton throughout Britain, but describes their species identity as being "in a state of chaos." The aims of this chapter are to review the *Obelia* species that have been identified in the past by hydrozoan experts, and to establish if fluorescence and bioluminescence can be used to rapidly distinguish the supposed different *Obelia* species from one

another. In addition the use of fluorescence and bioluminescence to easily distinguish non luminous from taxonomically similar luminous species will be investigated.

3.1.2 The discovery and naming of *Obelia* species

Linnaeus named the species of *Obelia geniculata* and *Obelia dichotoma* in 1758 (Linnaeus 1758). The name *Obelia* originates from the Greek for needle, whereas *geniculata* means sharply bent like a knee. *Dichotoma* also originates from the Greek word meaning "cut in pairs" (www.dictionary.com). Pallas identified *Obelia longissima* in 1766 and the fourth *Obelia* species *Obelia bidentata* was named by Clarke in 1875. These four species of *Obelia* are described in Paul Cornelius's North West European thecate hydroids and their medusae (Cornelius 1995), but he notes the difficulties encountered in distinguishing them from one another (refer to section 3.1.7).

The genus *Obelia* was assigned to the medusa by Peron and Leuseur in 1809 (Peron and Leuseur 1809; Hincks 1868).

Edward Forbes made a relatively early observation of an *Obelia* medusa in, 1848 he named it *Thaumantias lucifera* and described it thus, "the most phosphorescent of all the naked-eye species". He also noted that its tentacles were close set in a series, and that several of the medusae had no fewer than 84 in number (Forbes 1848).

Cornelius describes two species of *Obelia* medusae which have been observed in plankton samples from the British Isles. Distinguished from one another by

tentacle number, umbrella diameter and position of gonads on the radial canals, the smaller medusa type with less than 100 tentacles has been termed *Obelia lucifera* as named by Browne in 1905 (Browne, 1905). This medusa has now been reared from culture of hydroid colonies of both *Obelia dichotoma* and *Obelia geniculata.* The second medusa type can have up to 200 tentacles and darkly covered basal bulbs. It has been called *Obelia nigra or Obelia fimbriata.* This has been shown to arise from *Obelia longissima.* Mammon (1965) harvested medusae from *Obelia bidentata* but did not keep them alive for more than a few days.

Therefore it is possible that there are 3 species of *Obelia* medusae (Cornelius, 1995).Whether the medusa is named after the hydroid it originates from, or takes its own individual name is still being debated and is another source of confusion. Table 3.1 shows the different names that have been used in the past for *Obelia's* medusae adapted from an original list by Russell.

Name	Author
Thaumantias lucifera	Forbes, 1848, *Monograph, Briish Medusae, p.52, plate .x, fig.2.*
Laomedea geniculata	Gosse, 1853, *Devonshire Coast , p. 84, pl iv*
Obelia (Laomedea) geniculata	Allman, 1867, *Rep. Brit. Association .p. 77.*
Obelia geniculata	Hincks, 1868, *Brit. Hydroid Zoophytes, p. 149, pl. xxv,fig.1*
Obelia dichotoma	Hincks, 1868, *ibid. p. 156, plate .xxviii*
Obelia nigra	Browne, 1900, *Proceedings R Irish Academy series. 3, vol. v , p 721*
Obelia lucifera	Browne, 1905, *ibid. p. 770*
Obelia spp	Kramp, 1927, *K. danske vidensk. Selsk. Skr .natur. og math. Afd, 8. Raekke, xII, p. 124.*

Table.3.1. Taxonomic names used for *Obelia's* British medusa. This is adapted from an original list compiled by Russell (Russell, 1953).

3.1.3. Cnidarians

This phylum includes the Hydroida, Anthozoa and Schyphozoa. Cnidarians have two main life stages in their reproductive cycle, the sessile cylindrical polyp and a free swimming pelagic medusa. In the different classes of Cnidarians the dominance of these two life forms differs. The Hydroida are a class of aquatic organisms where the free swimming medusa stage is much smaller than the hydroid (polyp) stage. In this class the larger long lived colonial hydroid stage dominates (Hayward and Ryland, 1994).

Hydroids are one stage in the life cycle of the hydrozoa. All the *Obelia* hydroid species are thecate hydroids. These hydroids have tentacle bearing feeding polyps encased in a protective capsule called hydrothecae e.g. *Obelia geniculata.* Athecata hydroids don't have hydrotheca e.g. *Velella velella* commonly known as the by 'the wind sailor'**.** In addition to hydrothecae, most thecate hydroids have reproductive structures called gonothecae as well. Blastostyle cells within the gonothecae produce medusae by asexual budding.

The Scyphozoa class of Cnidarians exhibit a dominant medusa stage; hence these organisms are commonly termed jelly-fish, whereas in the Anthozoa class only the sessile polyp stage is seen. Scyphozoans only live in marine environments. The upper surface of these "jelly-fish" is called the exumbrella and the lower surface is the subumbrella. Unlike the medusae originating from hydroids, the Scyphozoan subumbrella has no vellum. Four septa divide the inner colenteron into a stomach and several radial pouches. "The mouth occurs at the end of a tubular mannbrium" (Hayward and Ryland, 1994), and is surrounded by tentacles. Marginal tentacles

70

often hang from the edge of the jelly-fish's bell and contain cnidocytes. Typical members of this class include the bioluminescent species *Pelagia noctiluca* and *Periphylla periphylla* (their bioluminescence is discussed in chapter one).

The sessile Anthozoans have an inner colenteron divided by six or more septa which terminates in a mouth often surrounded by tentacles. They can be solitary or colonial (Hayward and Ryland, 1994). This class includes corals, sea pens, sea fans and anemones. *Renilla remiformis* and *Pennatula phosphorea* are typical examples of bioluminescent Anthozoans (their bioluminescence is discussed in chapter one).

3.1.4 Previously reported habitats of the *Obelia species.*

Obelia geniculata is nearly always found on the large brown seaweed *Laminaria.* Johnston described *Obelia geniculata'*s habitat as seaweeds that grow near low tide mark and noted that it appeared as a network growing over fronds of *Laminaria digitata* (Johnston, 1847). The description of its habitat from more modern researchers is very similar and they are all in agreement, that *Obelia geniculata* is found on algae mainly *on Laminaria.* It prefers to remain submerged growing sub littoral and is nearly cosmopolitan on British coasts (Hayward & Ryland, 1994; Morin & Hastings, 1971a). It lies dormant in the depths in colder months and new colonies begin to appear on *Laminaria* in mid June. In British waters medusae are released in summer. In contrast *Obelia longissima* occurs on plants, rocks, sand or other substrata. It is thought to be nearly cosmopolitan on British coastlines. Morin and Hastings found specimens of *Obelia longissima* for their research on marine piles. Medusae are released from the British *Obelia*

longissima colonies in spring (Hayward & Ryland, 1994; Morin & Hastings, 1971a).

3.1.5 Life cycle and development

The reproductive life cycle of *Obelia geniculata* (refer to Fig.3.1) involves, medusae of approximately 5mm width, being released from the gonothecae which may be male or female. They are released into the plankton during spring or summer. Once in the plankton it takes several months for them to grow and sexually mature. Gonads appear as four circles of tissue on their radial canals (Vines & Rees, 1968). Female medusae produce eggs by meiosis whereas male medusae release sperm into the sea. Fertilisation occurs and the embryo thus formed "develops into a planula" (Cornelius, 1995).

Limited studies have been conducted on planula, but these suggest that they exist in the plankton for a maximum time of a few days (Cornelius, 1992). The planula is the smallest stage of the hydroid life cycle being smaller than 1mm.

The planula settles on a suitable substrate and in *Obelia geniculata's* case this is normally *Laminaria*. It then commences its metamorphosis into the *Obelia geniculata* colony. Its metamorphosis is by asexual cell division as is the subsequent growth of the hydroid colony. This life cycle is an example of metagenesis which means the "deferment of the power of generation" because the hydroid itself does not produce the sexual gametes (Vines & Rees, 1968).

3.1.6 Structure

All Cnidarians are aquatic organisms which exhibit radial symmetry and have a single body cavity called a coelenteron. The sac shaped coelenteron has only one opening which acts as both mouth and anus (Hayward et al. 1996). They have an outer body layer called the ectoderm and an inner layer the endoderm. The mesogloea exists between these two layers and in most medusae forms the transparent jelly.
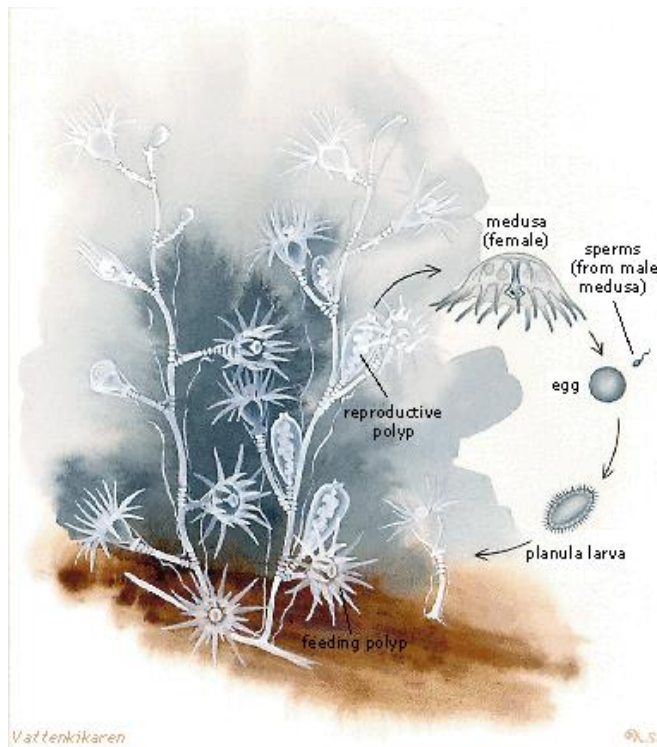
Fig.3.1. The life cycle of *Obelia geniculata*.

http:/www.vattenkikaren.gu.se/fakta/arter/cnidaria/hydrozoa/obellive.html. Author Tjarno Marine Biological Laboratory. Accessed 05.01.2011 at 7.33am.

The hydroid has an outer perisarc (secreted from its ectoderm), and an inner coenosarc, which encloses the 3 layers, ectoderm, mesoglea and endoderm. The mesoglea is a very thin non-cellular, jelly like layer (Hayward & Ryland, 1994;

Vines & Rees, 1968). This reflects the typical diploblastic structure of all Cnidarians (Hartog et al. 1906).

The structure of the perisarc is complex but its main components are chitin and proteins. Its branching tube forms an exoskeleton giving both protection and support to the hydroid (Bouillon et al. 1992). The ectoderm of *Obelia* also contains some sensory cells, its nervous system is discussed in section 4.1 (Green et al. 1990). Individual hydroids in an upright form are joined at the base by "a living tube of tissue" called the stolon. The stolon anchors the hydroid colony to its preferred substrate e.g. seaweed or rock. Upright stems arise from the stolon of the hydroid in *Obelia geniculata* these are approximately 3cm long. The hydroid colony originally arises from the asexual division of a single planula (Cornelius, 1995). The mouth of the hydroid (which also acts as its anus) opens out onto the hypostome. A ring of tentacles with cnidocytes lie beneath the hypostome. Cnidocytes on the tentacles can sting passing prey to paralyse them. Cnidocytes are a feature common to all Cnidarians, they are stinging cells normally borne on tentacles. Cnidocytes contain nematocysts (cnidae) which have coiled threads with barbs contained in a capsule.

Nematocysts are fired at prey as they float past in the plankton, or used as a defence mechanism. The sting of some Cnidarians nematocysts can be highly toxic e.g. *Physalia physalis* more commonly known as the Portuguese man-o-wars (Hayward et al. 1996).

 Prey consisting of microscopic zooplankton, are taken into the mouth and some digestion occurs in the hydranth (Cornelius, 1995). Particles of digested food are

eventually taken down into the coelenteron cavity within the hydroid. This cavity is continuous from one upright polyp to another so that by wafting of cilia lining the cavity food particles can be transported and nutrients can be shared within the colony (Morin & Cooke, 1971a). Glandular cells lining the coelenteron secrete digestive enzymes, resulting in some extra cellular digestion. Pseudopodial cells ingest food particles by phagocytosis. Once inside the cell more intracellular digestion occurs (Green et al. 1990).

Fig.3.2.The Structure of *Obelia geniculata*.The image shows the feeding tentacles and hypostome. It also illustrates the position of pedicles and a gonotheca, which contains medusa. The pink colouration is due to the presence of symbiotic red algae.

3.1.7 Bioluminescence

3.1.7.1 Photocyte cells in *Obelia*

Photocytes are specialised cells in *Obelia* which produce bioluminescence. Under the fluorescent microscope the position of *Obelia geniculata's* photocytes can be clearly seen as they contain GFP. Titschack worked on the pennatulid *Veretillum* in the 1960's and suggested that fluorescence he observed was associated with areas of bioluminescence (Titschack, 1964). Hastings and Morin went on to define photocytes as cells specialised for light emission and were the first to firmly establish that *Obelia geniculata's* photocytes all contain GFP. They achieved this by using fluorescent microscopy and image intensification simultaneously. They found that the photocytes are 10 to 20 μm in diameter. They found that the hydroid's bioluminescence was produced in photocyte cells in the endodermis of the hydroids stem and noted that there were no photocytes in Obelia's hydranths (Hastings, 1996, Hastings & Morin, 1998). Morin and Hastings observed photocytes in live *Obelia* specimens and some that were freeze dried .They reported that some photocytes had "a projection extending towards the colenteron" (Morin, 1974). They also succeeded in isolating membrane bound fluorescent particles from photocytes (Morin & Hastings, 1976). In the 1970's Morin and Hastings found that *Obelia geniculata's* bioluminescence could be initiated by mechanical, chemical or electrical stimulation. They described the resulting light emission as "a volley of 3 to 10 facilitating flashes each having a duration of about 75 milliseconds at 20^0C "(Morin & Hastings, 1971a).

Both fluorescence and bioluminescence are exhibited by *Obelia's* photocytes and the difference between these two types of light emission has already been discussed in chapter one. The fluorescent images of different species of *Obelia* reveal a different location of photocytes in at least three of the species, which can significantly aid their accurate identification. This is discussed in detail in section 3.1.8.

3.1.7.2 Obelin

The photoprotein obelin is contained within photocyte cells Obelin is triggered when Ca^{2+} bind to it producing a very rapid flash of bioluminescence.

Obelin contains coelenterazine linked to apo obelin by a covalent bond.

Within the obelin complex coelenterazine is in its peroxidised form and it acts as the most reactive part of the photoprotein (Shimomura, 2006).

The presence of obelin in living hydroids was discovered by Morin and Hastings in 1971 (Morin & Hastings, 1971a). They extracted photoproteins from a range of Cnidarians including *Obelia*. They extracted obelin from the four different species of *Obelia* and partially purified the resultant photoproteins.

They measured the emission spectra of the obelins and found that the maximum wavelength (λ max) was identical for the photoproteins from *Obelia geniculata* and *Obelia longissima* i.e. 475 nm.

They measured the rate constants for the bioluminescence produced by the photoproteins of the different species; comparing in vivo measurements with in vitro (refer to Table 3.2).

Cnidarian species	*In vivo* rate constant k sec^{-1}	*In vitro* rate constant k sec^{-1}
Obelia geniculata	54	3.4
Obelia longissima	50	0.6
Obelia commensuralis	58	1.6
Obelia bicuspidata(bidentata)	74	0.49
Aequorea forskalea	9.9	1.2
Clytia edwardsi	44	0.4

Table.3.2. The Properties of Cnidarian biouminescence. Adapted from Morin and Hastings 1971(Morin & Hastings, 1971a).

This showed that the in *vitro* rate constant for *Obelia geniculata* is similar to that of *Mnemiopsis leidyi* (a species of comb jelly), but far greater than that for aequorin. Differences in rate constants of photoproteins between some Cnidarian species may indicate that they evolved relatively early and have subsequently evolved some distinct differences. The far more rapid rate of bioluminescence in vivo suggests that photocytes exert control over Ca^{2+} input and its eventual rapid removal. Some Cnidarian's must control the reaction in a different way because the scyphozoan *Pelagia notiluca* uses a photoprotein and Ca^{2+} to produce a luminescent glow rather than a flash (Morin & Hastings, 1971a).

Morin & Hastings also proved that the bioluminescent reaction of these photoproteins is not dependant on the presence of oxygen and proceeds in anaerobic conditions. This agrees with the findings of previous researchers including Harvey and Shimomura (Harvey 1926; Shimomura et al. 1962). This

illustrates a key difference between photoprotein bioluminescence and bioluminescence produced by a luciferin luciferase system.

McElroy and Seliger (1963) proposed that aequorin was relatively stable peroxide. Morin and Hastings supported this view with their hypothesis that Cnidarian photoproteins are "relatively stable enzyme-substrate compounds".

Shimomura considers the light emission chemistry of obelin to closely resemble that of aequorin, "the chemical mechanism of obelin luminescence is considered to be identical with that of aequorin" (Shimomura, 2006).

Shimomura's research and the work of Morin and Hastings and research led by A.K.Campbell showed that these two photoproteins respond in an almost identical way to the addition of various metal ions. Both photoproteins react rapidly with Ca^{2+} producing light. The nature of the anion does not effect this reaction i.e. calcium acetate produces the same magnitude of light as calcium chloride. Strontium does initiate a bioluminescent response from the photoprotein, but this is only a hundredth of the magnitude of the response initiated by calcium. Magnesium ions have been shown to inhibit the bioluminescence of both photoproteins. Potassium, zinc, copper and iron ions elicit no response from either of the photoproteins. Cadmium ions (Cd^{2+}) will also trigger the luminescence of both aequorin and obelin (Bonder et al. 1995; Trofimov et al. 1994).

Both the photoproteins will also bind EGTA at the calcium binding sites thus inhibiting their luminescence (Shimomura, 2006; Campbell, 1974). The characteristics of obelin were investigated by Campbell in 1974. He confirmed that it will produce light within 100ms when reacted with Ca^{2+}. He found the

optimum pH for obelin extraction is pH 7.5 and that the photoprotein can be used to measure changes in intracellular Ca^{2+}. At the time this was very important as recombinant aequorin and obelin were not available to researchers. Moisescu, Ashley and Campbell discovered that obelin reacts faster as a calcium reporter than aequorin and is less affected by Mg^{2+}. However obelin is less sensitive than aequorin at lower calcium ion concentrations (Moisescu et al. 1975; Sala-Newby et al. 2000). D.G.Stephenson & Sutherland in 1981 extracted obelin from *Obelia geniculata* and *Obelia australis.* They deduced a molecular weight for obelin between 21,000 and 24,000 (Stephenson & Sutherland, 1981).

An important milestone was achieved when the c-DNA coding for apo-obelin was extracted and sequenced from *Obelia longissima* by B.A.Ilarionov (Ilarionov et al, 1995). Not surprisingly the size of the apo protein was similar to that of apo aequorin. It was made up 195 amino acid residues and had a molecular mass of 22.2 KDa. The c-DNA sequence was then utilised to produce recombinant apoobelin in *E. coli*. X-ray crystallography has now proved that obelins are 3D globular structures with 4 E-F hand Ca^{2+} binding sites. The hydrophobic core of the photoprotein contains the peroxidised coelenterazine (Vysotski et al. 1999). S.V.Markova cloned the c-DNA for obelin from *Obelia geniculata* and found that it had an 86% sequence similarity with that for obelin from *Obelia longissima*, and 64% similarity with that of aequorin. This degree of similarity reflects the fact that they are all calcium binding photoproteins. Markova also established that there were differences in the emission spectra obtained from natural obelins compared to recombinant obelins. λ max for the natural obelins is 475nm, whereas the

recombinant *Obelia geniculata's* obelin has a λ max at 495nm and *Obelia longissima's* obelin has a λ max at 485nm (Markova et al. 2002).

3.1.8 Confusion over identifying the different species of *Obelia.*

Thomas Hincks in his work on British hydroid zoophytes in1868, observed that the Campanularidae family, to which *Obelia* belongs is

"Pre-eminent for delicate beauty and graceful habit"

Hincks describes the bell shaped hydrothecae of this family as being like crystalline chalices. He reported on a possible six species of *Obelia: - Obelia geniculata, Obelia gelatinosa, Obelia longissima, Obelia dichotoma, Obelia plicata and Obelia flabellata.* He observed *Obelia flabellata* in rock pools at Tenby and considered that it may be a variety of *Obelia dichotoma.* He stated that *Obelia plicata* only occurred in the Shetland Isles. Hincks noted: - *Obelia geniculata's* phosphorescence describing it thus "If agitated in the dark, a bluish light runs along each stem, flashing fitfully from point to point as each polypite lights up its little lamp" (Hincks, 1868). G J Allman in 1871 provided illustrations of the features of *Obelia's* medusae and observed how the cells of the medusa's inner lining were rapidly dividing (Allman, 1871). Pennington in 1885 gives a similar description of *Obelia geniculata's* phosphorescence and records the same six species as Hincks. He describes finding a "forest like mass" of *Obelia* hydroids on *Laminaria* (Pennington, 1885). In 1906 Hartog classified *Obelia* as a coelenterate, but placed it in the Eucopidae family of hydroids and not the Campanularridae. He also placed the luminous hydroid *Clytia johnstoni* in the Eucopidae together with its medusa *Phialidium temporarium.*

F.S.Russell described three species of *Obelia* hydroids in his book the medusae of the British Isles (Russell, 1953). His illustration of the three *Obelia* species is shown in Fig 3.3.

He considered that it was not possible to distinguish between the two proposed species of *Obelia* medusae that other authors had observed. *Obelia lucifera,* that was thought to arise from *Obelia geniculata,* and *Obelia dichotoma*, and *Obelia nigra* that was presumed to arise from *Obelia longissima.* Russell was unsure as to whether any of the hydroids or medusae were in fact separate species. Kramp published his synopsis of the medusae of the world in 1961; in this he gives numerous references for the recording of the *Obelia* species since 1910.

HYDROIDS. (Text-fig. 185 A–C.) *Obelia geniculata* (L.); *O. dichotoma* (L.); *O. longissima* (Pallas).

(For full descriptions see Hincks, 1868, *Brit. Hydroid Zooph.* pp. 149, 156, 154; pl. xxv, fig. 1, pl. xxviii, fig. 1, and pl. xxvii.)

Text-fig. 185. Hydroids of *Obelia* medusae. A, *Obelia geniculata*; B, *Obelia dichotoma*; C, *Obelia longissima*. (After Hincks, 1868, pl. xxv, fig. 1 a; pl. xxviii, fig. 1 a; and pl. xxvii, fig. b.)

Obelia geniculata. Stem zigzag, sometimes slightly branched, jointed at each of the flexures and thickened immediately below them, forming a series of projections from which pedicels arise. Hydrothecae somewhat obconical, slightly longer than wide, margin plain, borne on short, sub-erect, slightly tapering, annulated stalks with four to six rings.

Gonothecae axillary, urn-shaped, attached by short ringed stalk with three to four rings; each with several medusa buds.

Fig.3.3. The 3 species of *Obelia*. F.S.Russell's diagram of what he considered to be the 3 species of *Obelia* (Russell, 1953). These are not a true representation of the species as they exist in the wild. Firstly the feeding tentacles are not shown and secondly the photocytes are not represented. This thesis shows that the positions of the photocytes are definitive criteria for identification.

Kramp quotes over 20 different names that have been assigned to the species including: *Obelia pyriformis, Obelia hyalina, Obelia lucifera and Obelia adriatica* (Kramp, 1961). Morin working in the 1970's in the USA identified five species of hydroids: *Obelia geniculata, Obelia dichotoma, Obelia longissima, Obelia bicuspidata* and *Obelia commisuralis* (Morin, 1974). Since the 1990's authors were still referring to similar difficulties in distinguishing the four species of Obelia hydroids: *Obelia geniculata, Obelia dichotoma, Obelia longissima and Obelia bidentata* (also known as *bicuspidata* by some authors). Paul Cornelius has produced taxonomic keys to distinguish the four species of hydroid as have John Ryland and Peter Hayward (Cornelius, 1995; Hayward & Ryland, 1994). Ryland and Hayward state that there is a large amount of morphological variation within some species of Campanulinidae, and that young colonies of hydroids may lack some characteristics of older colonies, making them difficult to identify (Hayward & Ryland, 1990). Paul Cornelius states that "there is great phenotypic variation. Colonies of a species taken from different localities can look quite different, causing difficulties in their identification". However he states that "there is far less phenotypic variation in the *Obelia* medusae". He provides a useful pictorial key to identify medusae based on the original work of Russell (Russell, 1953). Cornelius regards studying gonothecae as not useful for identification, as these reproductive structures can vary at different times of the year.

Distinguishing the proposed four species of *Obelia* from one another has therefore remained a significant problem. It is vital to distinguish them correctly so that accurate analysis of their DNA can then be undertaken. They have also been confused with other taxonomically similar thecate hydroids e.g. the non luminous

Laomedea flexuosa is often confused with *Obelia geniculata.* This confusion is compounded by the fact that historically *Obelia geniculata* was named *Laomedea geniculata* and is recorded by the famous Victorian naturalist Philip Gosse under this classification in his book Tenby: a sea-side holiday (Gosse, 1856). Nematocysts of *Obelia* medusae and hydroids have been compared by Ostman (1987), but this is incomplete and complex work, particularly due to the fact that it is currently very difficult to reliably distinguish all four species of *Obelia* hydroids from one another. One component of my research has focused on using the measurement of bioluminescence and fluorescence together with taxonomic keys to accurately distinguish the *Obelia* hydroid species, from one another, but also from other taxonomically similar species.

3.1.9 GFP location

The presence and pattern of GFP in *Obelia's* hydroids and medusae may be a valuable tool to clearly distinguish between these supposed different species. It could form a rapid taxonomic tool. Using this technique followed by sequencing the DNA of all the different forms, would be the only way to confirm whether they are separate species. Hastings and Morin observed different positions of photocytes in different species of *Obelia describing* them as "dispersed in an upright of *Obelia geniculata*" and "concentrated in the tip of a pedicle" in *Obelia bidentata* (Hastings & Morin, 1998). Different patterns of photocytes may have evolved, due to *Obelia longissima* and *Obelia dichotoma,* deterring a specific predator from eating their hydranths. This predator may be a nudibranch. Different species of nudibranchs are known to eat different parts of the hydroid.

For marine biologists, observing this fluorescence could aid identification of the different species of *Obelia*. It also rapidly distinguishes *Obelia* species from the non luminous *Laemodea flexulosa* which in the past has often been misidentified as *Obelia*. It is a straight forward procedure to grow most species of hydroids in seawater tanks. However culturing *Obelia* species can be very problematic. In the past scientists have cultured what they thought was *Obelia* only to discover that it was in fact *Laemodea (*Crowell, 1953*)*. Table 3.3 states the current classification of *Obelia geniculata.*

Phylum	Cnidarian
Class	Hydrozoa
Order	Hydroida
Suborder	thecata (Leptomedusae)
Family	Campanularia
Genus	*Obelia*
Species	*geniculata*

Table.3.3. Classification of *Obelia geniculata* (Hayward and Ryland, 1994).

Species name	Author and date
Obelia geniculata	Linnaeus 1758
Obelia dichotoma	Linnaeus 1758
Obelia longissima	Pallas 1766
Obelia bidentata	Clark 1875
Obelia lucifera	Forbes 1848 *Monogr,Brit Medusae*
Obelia fimbriate	Dalyell 1848
Obelia plicata	Hincks, 1868, *Brit. Hydroid Zooph,*
Obelia longicyatha	Allman 1877
Obelia castellata	Clarke 1894
Obelia hyalina	Clarke 1879
Obelia dubia	Nutting 1901
Obelia obtusidens	Jaderholm 1904
Obelia paulensis	Vanhoffen 1910
Obelia multidentata	Fraser 1914
Obelia longo	Stechow 1925
Obelia alternata	Fraser 1938
Obelia biserialis	Fraser 1938
Obelia equilateralis	Fraser 1938
Obelia racemosa	Fraser 1941
Obelia irregularis	Fraser 1943

Table.3.4. Historical names given to *Obelia* species. http://www.encycopedia of life.org/pages/203009. Accessed 17.02.2011 at 8.15am.

3.1.10 Aims and strategy

Given the confusion over the taxonomy of the different species of *Obelia*, the fluorescence and bioluminescence of the species were investigated to ascertain if they could be utilised as a definitive indicator of species.

The experimental strategy involved:-

- Finding sample sites for the different species of *Obelia* in Pembrokeshire.

- Setting up sites for rapid sampling of hydroids in the Milford Haven area and testing to ensure these were not affected by pollution.

- Using the chemiluminometer to record the bioluminescent response of hydroid species as well as the activity of obelin extracts.

- Recording fluorescent photocyte patterns for the species of *Obelia* in Pembrokeshire and Plymouth.

- Extraction of photoprotein from *Obelia geniculata* and testing its activity.

- Developing a protocol to distinguish the 4 species of *Obelia* from one another.

- Developing a protocol to distinguish taxonomically similar non luminous hydroids from luminous species.

3.2 Methods

3.2.1 Measuring GPS

GPS positions were recorded using a Garmin 12 personal navigator supplied by Garmin International Inc. 1200E, 151 Street, Olathe Kansas 66062.

3.2.2 Bioluminescent marine organisms

Most samples were obtained in Pembrokeshire but some samples, were taken at Plymouth whist working at the Marine Biological Association (MBA), Citadel Hill Laboratory.

3.2.3 Sample sites in Plymouth.

Obelia geniculata was obtained from *Laminaria* below MTL in Plymouth sound very close to the MBA Citadel Hill laboratory. *Obelia longissima* was taken from sampling ropes at Queen Anne's Battery marina. This marina has no lock gate and therefore water salinity is identical to that of the sea. *Obelia dichotoma* was found growing on Ross coral (*Pentapora fascialis)* sublittorally in Plymouth sound.

3.2.4 Sample sites in Pembrokeshire

Easily accessible sample sites for the luminous hydroids *Obelia geniculata* and *Obelia longissima* had to be found in Pembrokeshire. Initially sites were studied as they were recorded in the Dale Fort marine fauna as containing *Obelia* colonies (Crothers, 1966). These records were compiled in the 1950's and 60's from a card index of reported marine organisms held at the field studies centre. *Obelia geniculata* was only found at two of these sites and *Obelia longissima* at one. All the sites yielded some hydroids but these were mainly either *Dynamena pumila* or

Laomedea flexuosa both of which are non-luminous. Some of the Dale Fort records suggested that *Obelia geniculata* could be found on the lower shore but this proved not to be the case. The features of the hydroids were studied under the microscope to determine their species. In addition bioluminescence of each sample of hydroids was tested using the chemiluminometer and fluorescence microscopy was used to detect the presence or absence of GFP in the hydroids.

The main hydroid sample sites in Pembrokeshire.
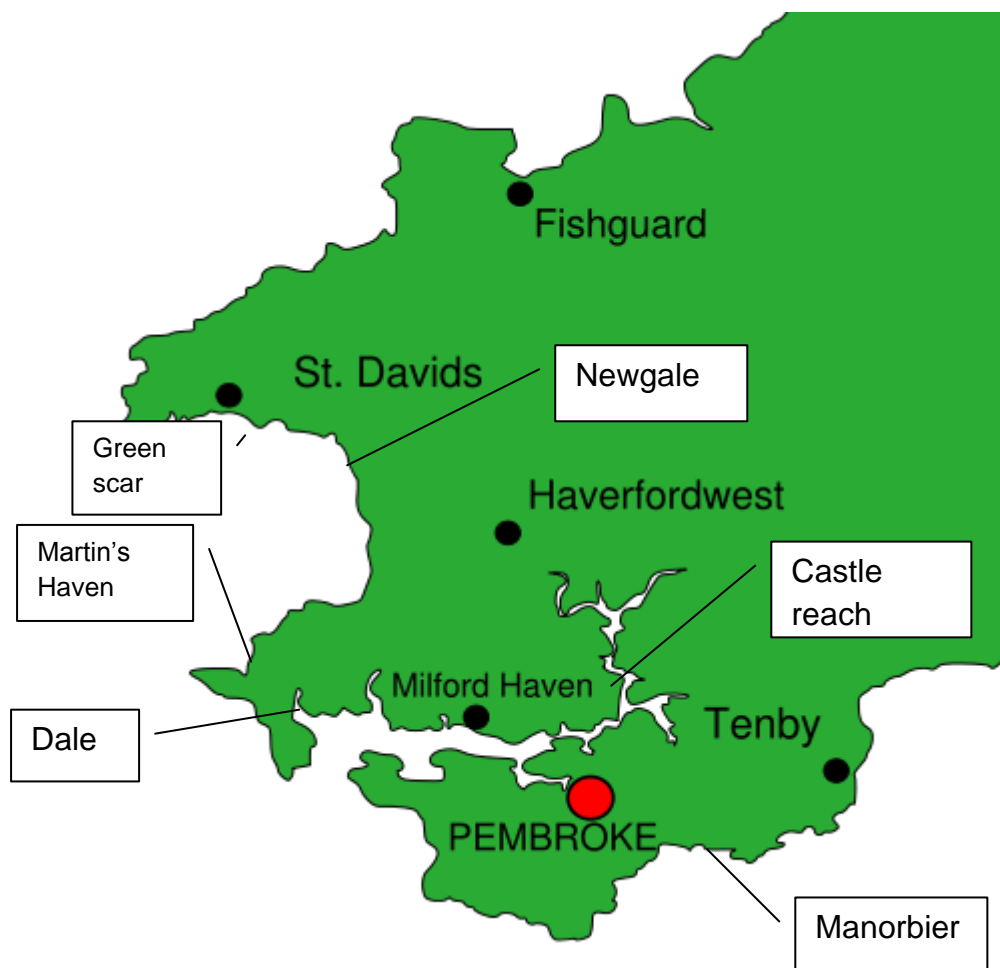
Fig.3.4.The main hydroid sample sites in Pembrokeshire. Full details of all Pembrokeshire sample sites including GPS locations are given in Table 3.5. (Adapted from www.Wikipedia.org.Accessed 9.3.12. 9am).

Many of the sites were very silted and only accessible on a very low tide. They were therefore not suitable as regular collection sites. Initially experiments were carried out using samples obtained by snorkelling at Martin's Haven. A license for collecting hydroids from within the Skomer marine nature reserve was obtained for this purpose. Some samples were also obtained from deep water by working with a local diver Steve Myatt. These samples were from Green Scar and Watwick point. It was established that in the summer months *Obelia geniculata* is ubiquitous on *Laminaria* in deep fast flowing water at many dive sites in Pembrokeshire, but going out with divers to collect samples proved very time consuming. Dale Rostrum kindly provided me with data from her collation of hydroid survey data in Pembrokeshire in the 1990's, but again it was found that most of the sites she recorded did not now contain *Obelia geniculata* but did have *Laomedea flexuosa.* These differences may be due to misidentification or dredging in these areas may have altered the species present and recording of exact positions of colonies may have been inaccurate. Both Dale Rostrum's work and the Dale Fort fauna records were constructed before the advent of GPS. To minimise the time spent on collecting hydroid samples sample sites were set up in Milford Haven close to a new marine research laboratory set up with the support of Milford Haven Port Authority (MHPA). The key conditions that colonies of *Obelia geniculata* seem to favour were considered, which are deep water (the hydroid does not like to be exposed except on occasional very low tides) strong current and abundant plankton. *Obelia geniculata* also prefers to grow on the seaweed *Laminaria.* This led to the discovery of a commercial jetty owned by MHPA, where *Obelia geniculata* grows on the *Laminaria* from mid June to late

October. Weighted marine plywood boards were positioned on ropes below the water at the MHPA jetty and also Milford Haven marina to ascertain if hydroids would attach to the boards and ropes. In the spring *Obelia longissima* grew on the marina board ropes but no luminous hydroid species grew on the boards at either location. The ropes in the marina provided a rapid sample point for *Obelia longissima* and a method for monitoring the same colony of hydroids in a controlled zone. *Obelia dichotoma* grew on the ropes in the late summer. Several species of non-luminous hydroids grew on the boards and ropes at the MHPA jetty together with other marine life, but *Obelia geniculata* did not.

Fig.3.5. Installing ropes & boards at Milford Haven marina. Boards were fitted in Milford marina in May 2008 and after one month barnacle napuli appeared on them. Within two months hydroids were growing on the ropes together with sea squirts. Simultaneously barnacles, sea squirts and other ascidians (sessile tunicates) had colonised the boards.

Fig.3.6. Marine organisms growing on rope, and sample board at Milford Haven marina.

Fig.3.7. *Obelia longissima* growing on rope attached to sampling board in Milford Haven marina.

3.2.5 Measuring water quality.

To establish that the 2 main sample sites for luminous hydroids were not affected by pollution, oxygen, nitrate, and coliform bacteria levels at both sites were measured together with the salinity and pH. As a positive control the same tests were also carried out at smoke stack quay and a local river running into the haven both of which are often polluted with sewage. The lovibond comparator and test kits were used to measure oxygen and nitrate levels full details are given in Chapter 2 section 2.4. A salt refractometer was used to measure salinity for details refer to chapter 2 section 2.4.3.

3.2.6 Measuring and photographing fluorescence

Fluorescence was measured and photographed from hydroid samples by the methods explained in chapter 2 sections 2.3 and 2.3.1. This includes an explanation of Fluorescence Lifetime Imaging Microscopy FLIM.

3.3 Results

3.3.1 Pembrokeshire hydroid sample sites.

Sample site	Hydroid species	GPS location	Luminous/non luminous
Black rock Dale	*Obelia geniculata*	Lat N 51º 42' 34.1" W005º 10' 06.0"	Luminous contains GFP
	Dynamena pumila	Lat N 51º 42' 34.5" W005º 10' 05.8"	Non luminous
Burton Ferry	*Obelia geniculata*	Lat N 51º 42' 27.7" W004º 55' 35.1"	Luminous contains GFP
	Dynamena pumila	Lat N 51º 42' 28.6" W004º 55' 34.5"	Non luminous
Castle Reach	*Dynamena pumila*	Lat N 51º 43' 30.6" W005º 6' 12.5"	Non luminous
	Obelia longissima	Lat N 51º 43' 30.6" W005º 6' 12.5"	Luminous contains GFP
Dale Beach	*Bougainvillia ramosa*	Lat N 51º 44' 29.6" W005º 10' 06.6"	Non luminous
	Laomedea flexuosa	Lat N 51º 44' 29.6" W005º 10' 06.2"	Non luminous
Garron pill	*Dynamena pumila*	Lat N 51º 43' 83.5" W004º 53' 01.2"	Non luminous
Green scar	*Obelia geniculata*	Lat N 51º 51' 39.0" W005º 12' 05.9"	Luminous contains GFP
Manorbier	*Obelia geniculata*	Lat N 51º 38' 31.6" W004º 48' 14.2"	Luminous contains GFP
Martin's Haven	*Obelia geniculata*	Lat N 51º 44' 10.3" W005º 14' 43.6"	Luminous contains GFP
Milford Haven marina	*Obelia longissima*	Lat N 51º 43' 45.5" W005º 02' 15.8"	Luminous contains GFP
	Obelia dichotoma	Lat N 51º 43' 45.5" W005º 02' 15.8"	Luminous contains GFP
Milford Haven Port Authority jetty	*Dynamena pumila*	Lat N 51º 42' 22.9" W005º 03' 04.3"	Non luminous
	Obelia geniculata	Lat N 51º 42' 23.1" W005º 03' 04.3"	Luminous contains GFP
	Obelia longissima	Lat N 51º 42' 23.1" W005º 03' 04.3"	Luminous contains GFP
	Tubularia indivisa	Lat N 51º 42' 23.1" W005º 03' 04.3"	Non luminous

Sample site	Hydroid species	GPS location	Luminous/non luminous.
Newgale	*Obelia geniculata*	Lat N 51° 50' 09.6" W005° 06' 52.1"	Luminous contains GFP
	Obelia dichotoma	Lat N 51° 50' 9.4" W005° 06' 51.8"	Luminous contains GFP
Sandy Haven	*Eudendrium ramosum*	Lat N 51° 43' 31.8" W004° 06' 15.8"	Non luminous
	Dynamena pumila	Lat N 51° 43' 31.8" W004° 06' 15.8"	Non luminous
St Catherine's Cave	*Dynamena pumila*	Lat N 51° 40' 15.6" W004° 41' 37.9"	Non luminous
Tenby	*Laomedea flexuosa*	Lat N 51° 40' 14.6" W004° 41' 34.1"	Non luminous
Tenby harbour	*Obelia dichotoma* Colony 1	N 51° 40' 20.3" W004° 41' 53.6"	Luminous contains GFP
	Obelia dichotoma Colony 2	N 51° 40' 21.6" W004° 41' 55.7"	Luminous contains GFP
Watwick point	*Obelia geniculata*	N 51° 41' 54.3" W005° 09' 07.8"	Luminous contains GFP

Table.3.6. Sample sites for hydroids identified in Pembrokeshire.

Twelve Pembrokeshire sites were found, which contained *Obelia* colonies. Three different species of *Obelia* were identified. Non luminous colonies of *Laomedea flexuosa* and *Dynamena pumila* were also found at some of the sites. These were used for comparison and controls. More easily accessible sample sites were established at Milford Haven with *Obelia longissima* and *Obelia dichotoma* growing on ropes set up in Milford Haven marina. *Obelia geniculata* was collected from *Laminaria* growing on the bottom of a commercial jetty at Milford Haven Port Authority. *Obelia bidentata* was not found in Pembrokeshire or at Plymouth, but it is known to prefer warmer water.

3.3.2 Water quality at key sample sites

Site name and location	Port authority jetty Lat N 51° 42' 23.1" W005^o 03' 04.3"	Smokehouse Quay Beach Lat N 51° 38' 31.8" W004^o 48' 13.4"	Milford Marina Lat N 51° 43' 45.5" W005^o 02' 15.8"	Haverfordwest River Lat N 51° 48' 17.3" W004^o 58' 12.7"
Mean ± Most probable number of coliforms per 100ml	3.6±0.04	75±0.35	3±0.07	1100±0.71
Mean ± Oxygen (mg/L)	9.00±0.35	12.00±0.20	8.10±0.20	11.00±0.04
Mean ± Nitrate (mg/L)	0.15±0.04	0.1±0.04	0.2±0.05	1.4±0.36
Mean ± Salinity ppT	37.5±0.35	35.83±0.54	32.33±0.74	0±0
Mean± pH	7.21±0.09	7.23±0.04	7.16±0.02	7.33±0.05

Table.3.7. Results of water quality testing. This showed there is no significant sewage pollution at these two main sample sites. However the salinity in the marina is lower than seawater. Smokehouse quay and Haverfordwest River were used as a comparison as they are known to sometimes be polluted with sewage.

3.3.3 Fluorescent photocyte patterns in *Obelia* species

These were recorded using epifluorescence for 3 *Obelia* species as well as the medusa *Obelia lucifera. Clytia hemisphaerica's* photocyte pattern was recorded for comparison. Figs 3.8 to 3.13 illustrate that the fluorescent pattern is different for the three *Obelia* species and can therefore be used as a rapid taxonomic tool. The same patterns were observed in specimens from Plymouth and Pembrokeshire. Fluorescence in the hydranths including the hypostome and

tentacles of *Obelia longissima* and *Obelia dichotoma* was observed. A FLIM analysis on *Obelia dichotoma* suggested this was due to the presence of GFP. Previous researchers have not observed photocytes or fluorescence in hydranths (Hastings, 1995).
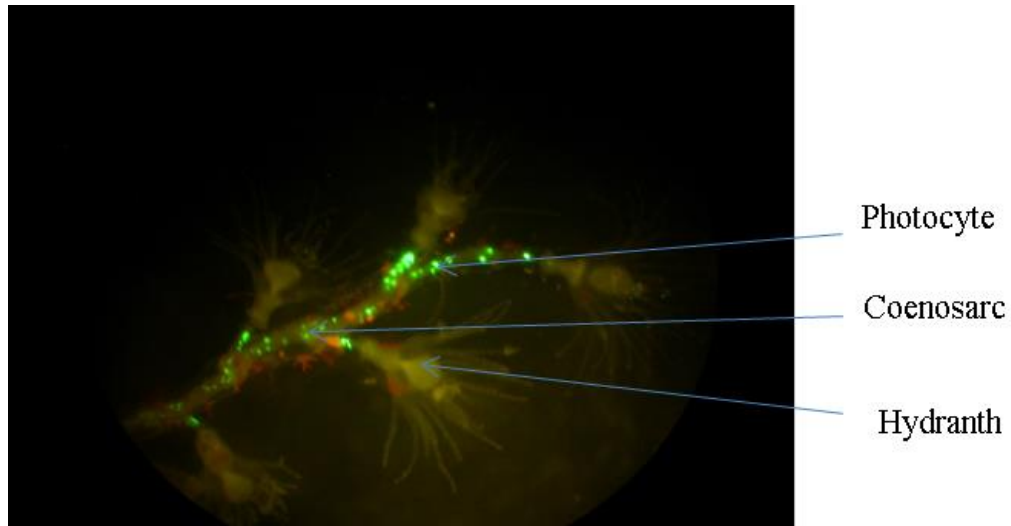
Fig.3.8. **Photocyte pattern in *Obelia geniculata*.** This was photographed using the Sentech camera and Hund H500 fluorescent microscope. Magnification X100. The image shows the photocytes relatively evenly dispersed throughout the coenosarc of the hydroid.
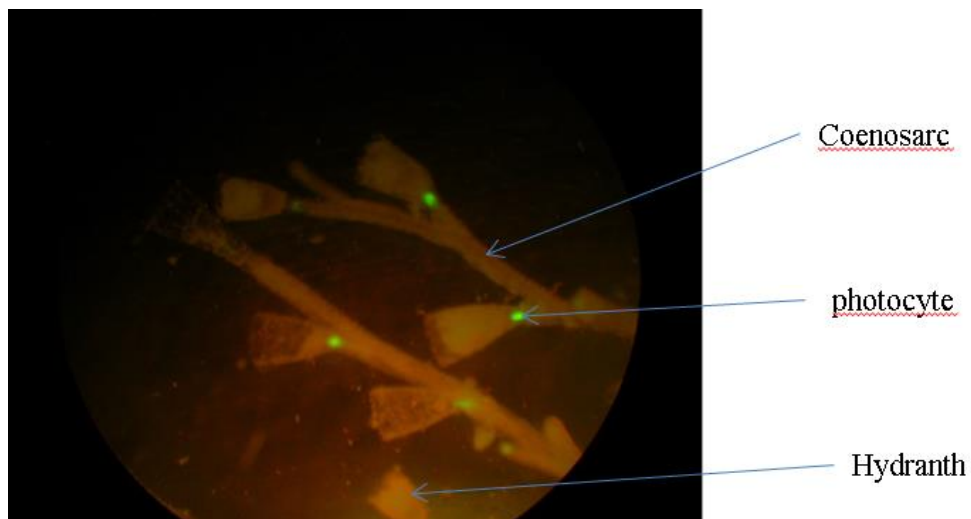
Fig.3.9. **Photocyte pattern in *Obelia longissima*.** This was photographed using the Sentech camera and Hund H500 fluorescent microscope. Magnification X100. A single photocyte exists at the base of each hydranth.

98
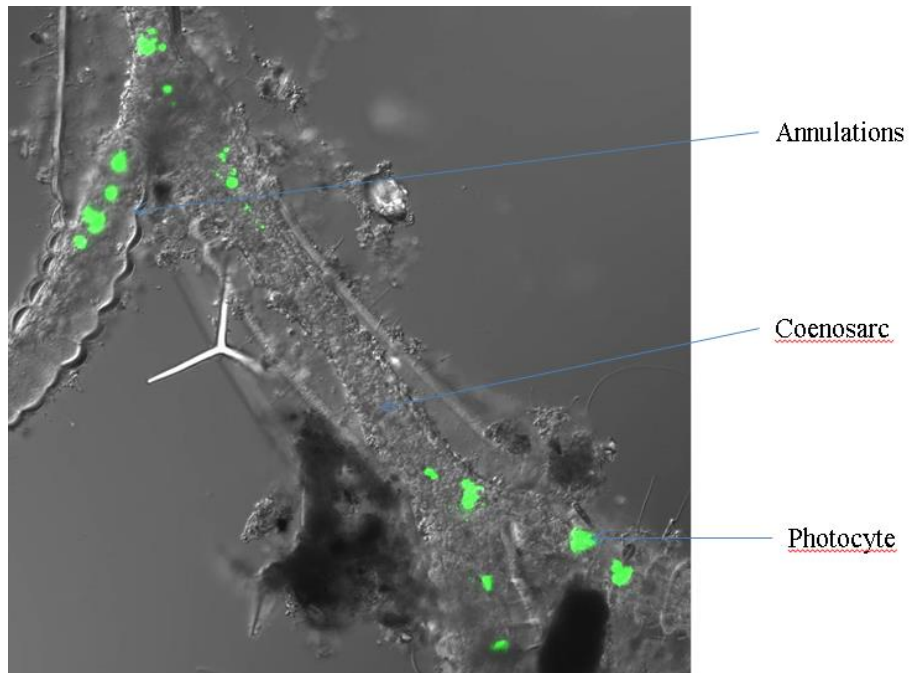
Fig.3.10 Photocyte pattern in *Obelia dichotoma*. The combined bright field and fluorescent image was recorded using the La vision biotech confocal microscope. Magnification X1000. The higher magnification was used to illustrate that the photocytes are not a uniform shape and although they are in the coenosarc they are mainly concentrated in the annulations, where the hydroid branches. The pattern is distinct from that of *Obelia geniculata*.
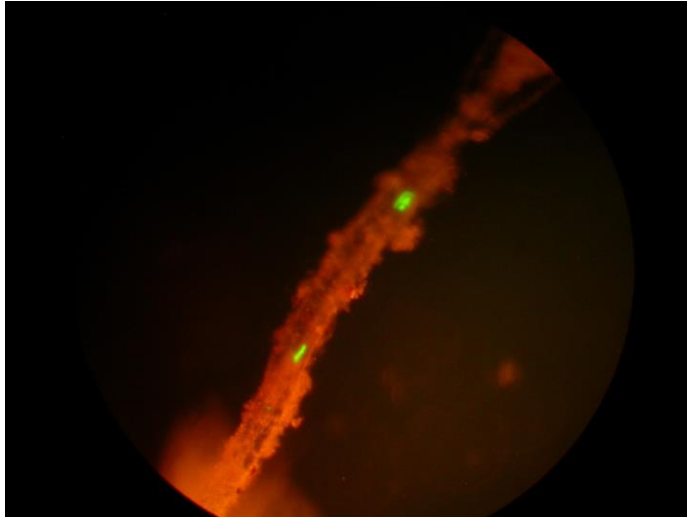
Fig.3.11. Photocyte pattern in *Clytia hemisphaerica*. This shows the combined bright field and fluorescence image of the photocyte pattern in *Clytia hemisphaerica.* The green fluorescent areas are the two photocytes in the hydroid's coenosarc. The red fluorescence is produced by red algae which exist symbiotically in the hydroid. The specimen was photographed using the Sentech camera and Hund H500 fluorescent microscope. Magnification was set at X100.
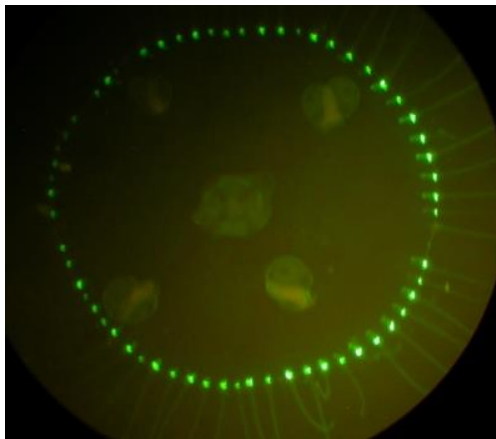
Fig.3.12. Photocyte pattern in *Obelia lucifera*. The green fluorescence is produced by tiny photocytes positioned around the rim of the medusa's umbrella, extending from them are the transparent tentacles. Specimen photographed with the Sentech camera and Hund H500 fluorescent microscope. Magnification X1000.
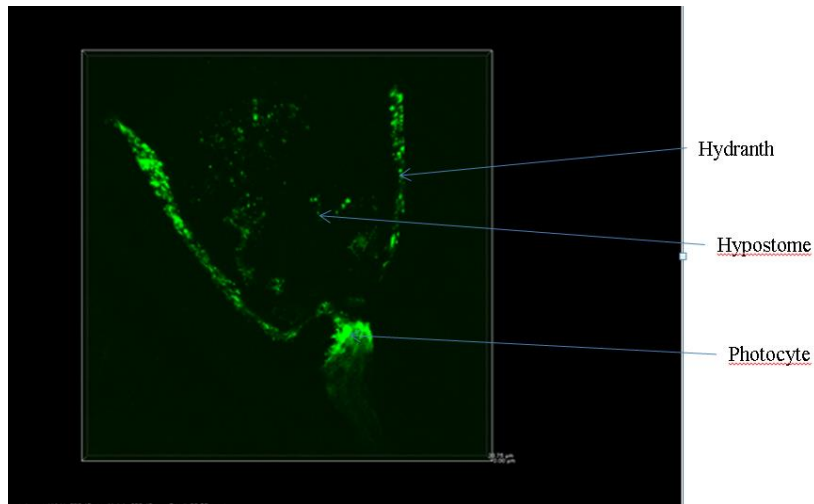
Fig.3.13.GFP in hydranth of *Obelia dichotoma*. Magnification x1000. Fluorescence in the hydranths, including the hypostome and tentacles of *Obelia dichotoma* was observed. The image was recorded using the La vision biotech confocal microscope, using magnification x1000. A FLIM analysis proved this fluorescence was due to GFP.

A FLIM analysis on this hydranth of *Obelia dichotoma* gave a fluorescent half-life of 3 nanoseconds which suggested this was due to the presence of GFP. Fluorescence was emitted at 525nm.The Image and analysis performed using the La vision biotech confocal FLIM. Interestingly the FLIM indicated the presence in smaller quantities of two additional unidentified fluors also in the hydranths. Previous researchers have not observed photocytes or fluorescence in hydranths (Hastings, 1995).

3.3.4 Bioluminescence of *Obelia*
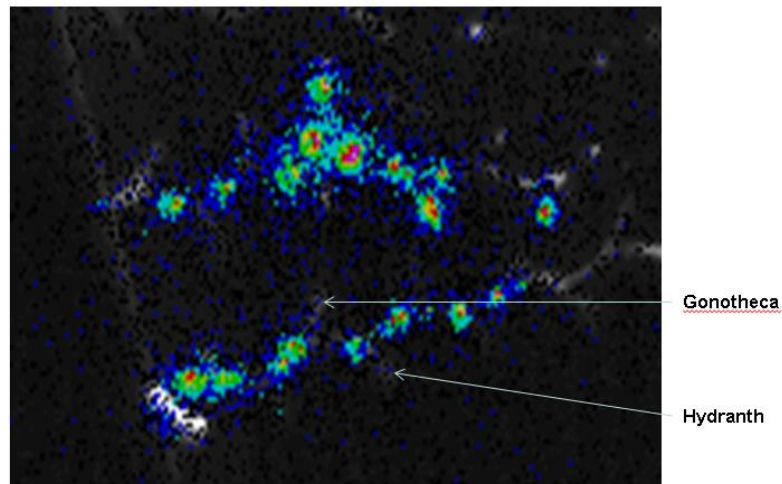
Fig.3.14. Bioluminescent photocytes of *Obelia geniculata*. The bioluminescence emitted from the photocytes of two hydroids, was recorded. Red areas show the position of the hydroids photocytes. Hydroid size 1.5cm pseudo colour image recorded with Photek imaging camera, and superimposed on bright field image. Bioluminescence stimulated by injection of 0.5M KCl. Total image recorded over 30 seconds. Red= bright light being emitted. Blue=dim light. Black=no light (Vassel et al. 2012).

This result illustrates that the bioluminescence is being produced in discrete areas i.e. the photocytes which contain the photoprotein obelin as well as GFP. Bioluminescence is not produced in the gonothecae or hydranths.

3.3.4 Bioluminescence of *Obelia* hydroids

Comparative bioluminescence of *Obelia* species
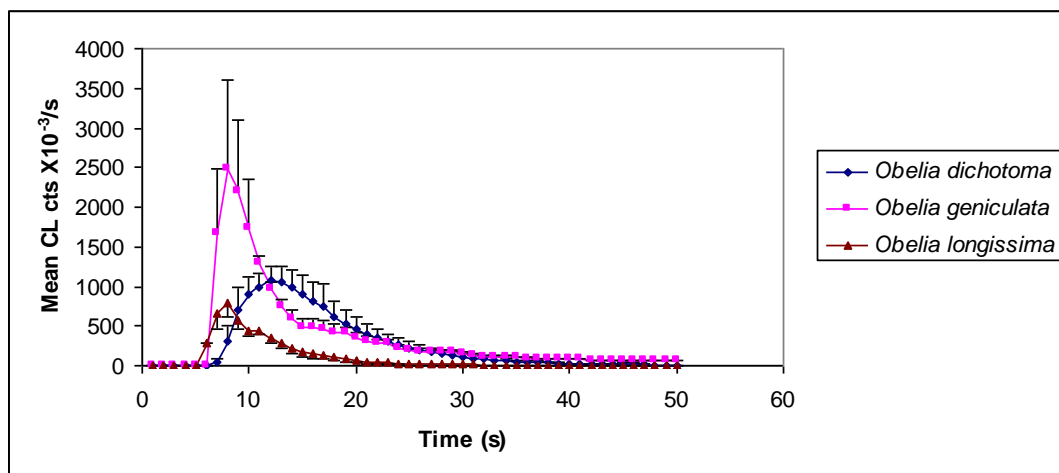
Fig.3.15. Comparative bioluminescence of *Obelia* species. Bioluminescence of the 3 species of *Obelia*. Bioluminescence of *Obelia* was stimulated by 1ml 0.5M KCl. Results represent the mean of six individual hydroids ±SEM.

The sample of *Obelia geniculata* was obtained from *Laminaria* on the Milford Haven Port Authority jetty. *Obelia dichotoma* was obtained from the siphon of a tube worm on Newgale beach. *Obelia longissima* was taken from sampling ropes in Milford Haven marina. Fig.3.15. illustrates that the mean bioluminescence of *Obelia geniculata* is twice that of the other two species of *Obelia* found in Pembrokeshire. *Obelia dichotoma's* response to K^+ stimulation was slower and broader than the other two species. *Obelia longissima's* bioluminescence was lower than that of the other two species, which could be due to the lower salinity of the water in the marina. Alternatively it may be due to the fact that *Obelia longissima* has less photocytes.

A comparision of the bioluminescence of *Obelia geniculata* with *Laomedea flexuosa*.
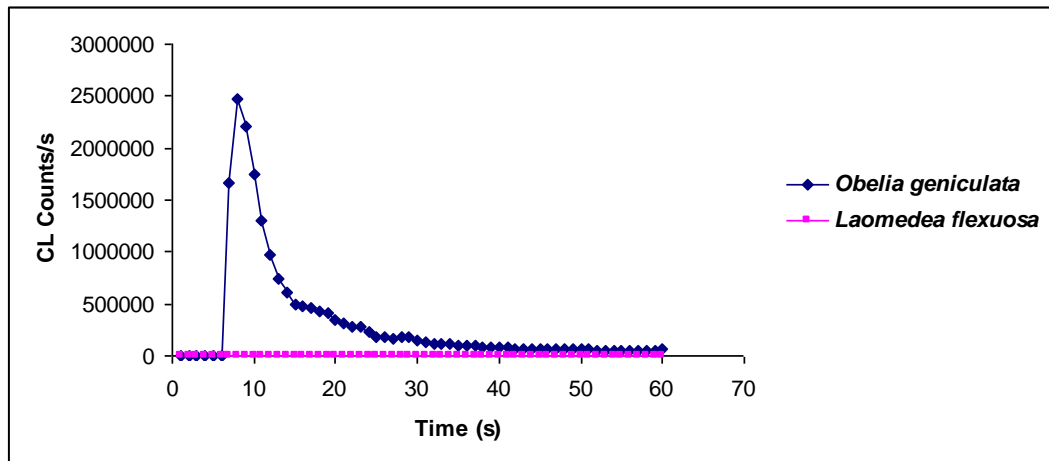
Fig.3.16. Comparison of bioluminescence of *Obelia geniculata* with that of the non-luminous *Laomedea flexuosa* from Dale beach. Mean counts per second were calculated from six individual species of each hydroid. This confirms that measuring mean bioluminescence with the chemiluminometer distinguishes the two taxonomically similar species.

Bioluminescence emitted from obelin
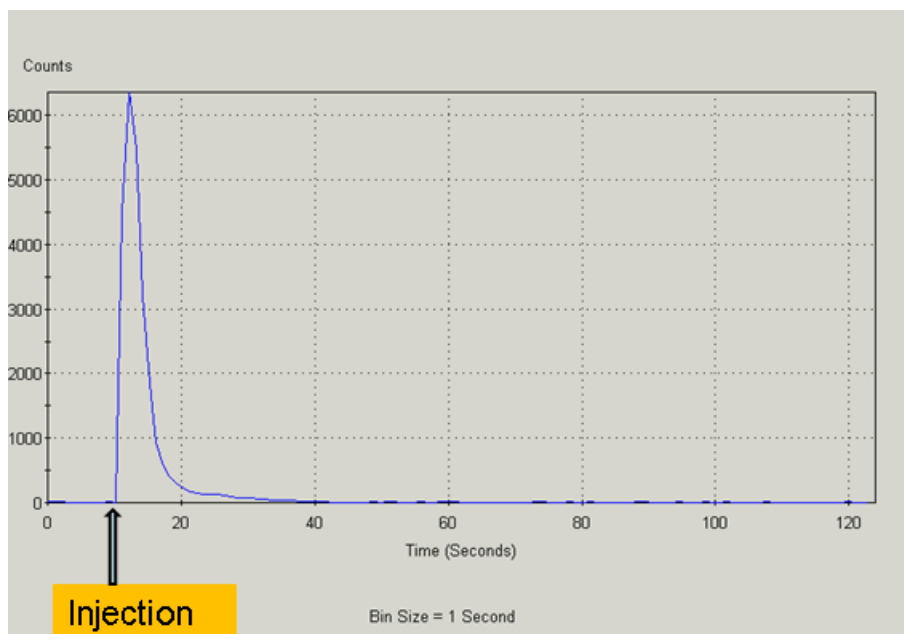
Fig.3.19. Bioluminescence emitted from obelin. Bioluminescence of obelin triggered by injection of 0.5 mls of 50 mM calcium ions at 10 seconds. This illustrates the rapid *in vitro* response of the photoprotein. The *in vivo* response is even more rapid. Bioluminescence was measured using the Photek imaging system, recording the light emission over 120 seconds.

Total image of light emitted from obelin.
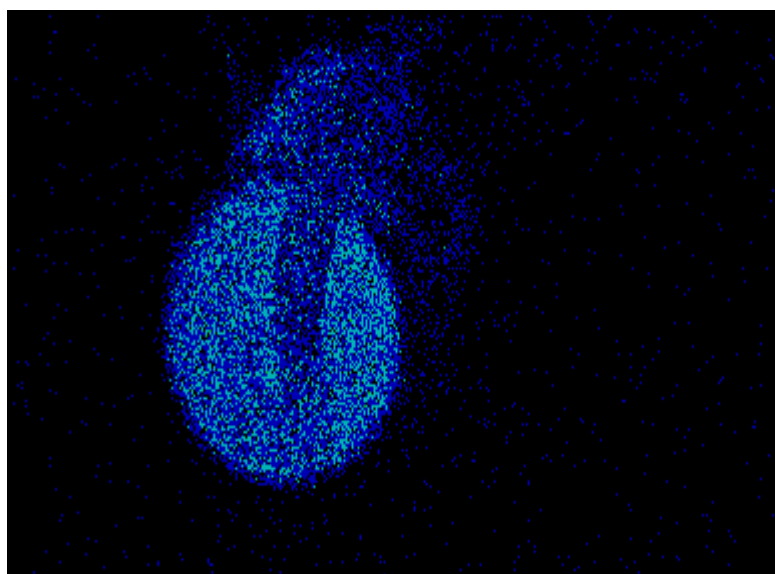
Fig.3.20. Total image of light emitted from obelin. Total image acquired when 50ul obelin was placed in one compartment of a multi welled plate. Bioluminescence triggered by injection of 0.5mls of 50 mM calcium ions at 10 seconds. Total image recorded over 120 seconds.
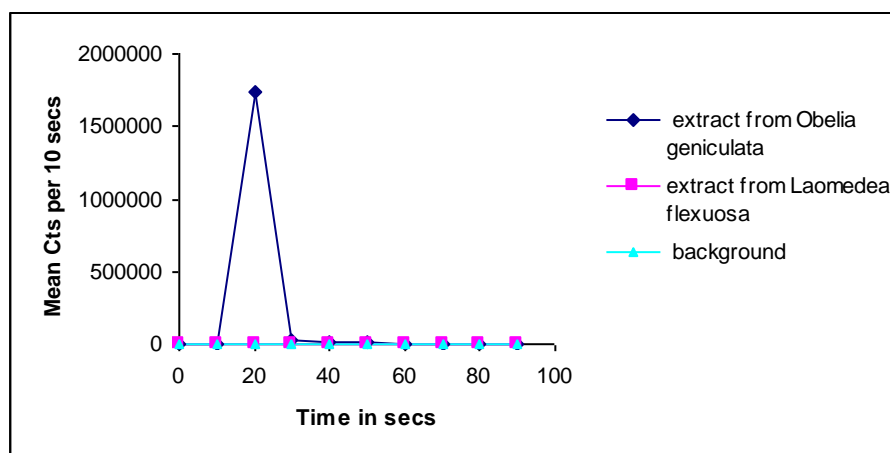
Fig.3.21. Comparing obelin activity. Obelin activity was compared in *Obelia geniculata* and *Laomedea flexuosa*. Bioluminescence of obelin triggered by injection of 0.5 mls of 50 mM calcium ions at 10 seconds. The activity of the obelin extract from *Obelia geniculata* was compared against an extract prepared from the non-luminous *Laomedea flexuosa* which was used as a control. The mean counts per 10 seconds were calculated from triplicate samples of each extract. This confirmed that non-luminous species, although taxonomically similar to *Obelia geniculata*, do not contain any photoprotein.

The effect on obelin activity of altering anion
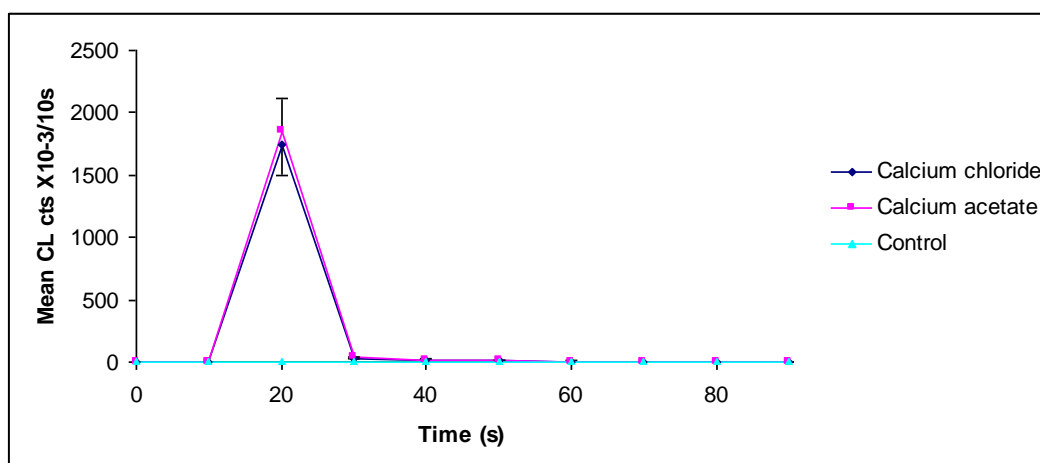
Fig.3.22. Effect on obelin activity of altering anion. The effect of using 0.5mls of 50mM calcium acetate to trigger obelin activity as opposed to 0.5mls of 50mM calcium chloride was measured. Mean counts per 10 seconds were calculated from triplicate obelin samples ± SEM. This illustrated that altering the anion in the calcium ions solution does not affect the amount of light produced by the obelin.

3.4 Discussion

The main conclusions of this chapter are that three species of *Obelia*, found in the Milford Haven waterway can be clearly identified. The results illustrate that fluorescent photocyte patterns, can be used to accurately and rapidly distinguish between the three species. Fluorescent photocyte patterns, together with bioluminescence measurement can be utilised to accurately distinguish between taxonomically similar luminous and non luminous species. Thus investigators can ensure that they are conducting their research on the correct species.

Four identification criteria have been established.

- Firstly measurement of bioluminescence using the chemiluminometer. Alternatively bioluminescence can be triggered in a dark room by squirting 0.5M KCl onto *Obelia*.

106

- Secondly use a fluorescent microscope to observe whether GFP is present. If present match the pattern of the fluorescence to those established for the three species of *Obelia*.

- Thirdly study structural characteristics under the microscope. *Obelia* has no annulations between segments. If gonothecae are present there are subtle differences to study in these.

- Fourthly carry out a photoprotein extraction. *Obelia* species will contain active photoprotein. Non-luminous species will not.

The results also illustrate that the habitats of the three species is important. Where a specimen is found and on what strata can greatly aid its identification. *Obelia geniculata* exists on sub littoral *Laminaria* and prefers to live where there is significant current. Whereas *Obelia dichotoma* is intertidal and sublitoral, it has been found on ropes, marine piles and on other living marine species. *Obelia longissima* is intertidal and sublitoral preferring slacker water. It has been found on ropes and algae and is often found in brackish water such as that which exists in Milford Haven marina.

Hayward describes *Obelia geniculata* as intertidal and sublittoral, but this is incorrect. No specimens were found above the tide line in Pembrokeshire or Plymouth (Hayward et al 1996). Hayward et al may have confused *Obelia geniculata* with the very similar *Obelia dichotoma* which can be found in intertidal rock pools. *Obelia geniculata* is found on sublittoral seaweed rarely exposed at low tide. Three different species of *Obelia* hydroids have been located at sample sites in Pembrokeshire and Plymouth. Main sample sites close to the

Milford Haven laboratory were utilised the most to ensure hydroid samples were as fresh as possible. These sites were tested to ensure they were not polluted. No significant pollution was found but it was established that the salinity in Milford Haven marina does vary from that of normal seawater. *Obelia bidentata* was not found in Pembrokeshire or Plymouth and this may be due to the fact that it prefers warmer waters. A new power station in the Milford Haven water way has already caused a rise in sea temperature in some parts of the haven and it will be interesting to see if this heralds the arrival of this fourth species in the haven. Similarly if global warming raises the temperature of the Haven, colonies of *Obelia bidentata* may then appear. Warmer waters may however have a detrimental effect on the existing three species.

3. 4. 1 Pembrokeshire habitats of *Obelia*

Obelia geniculata

Found on *Laminaria* at 8 sub littoral sites in Pembrokeshire. Two sites are in deeper water where samples were obtained by diving. The site at Milford Haven Port authority jetty, allows samples of *Obelia geniculata* to be obtained and utilised in the marine laboratory within one hour.

Obelia longissima

Found growing on ropes, mussel shells or *Laminaria* at 3 sites in Pembrokeshire. Most specimens were obtained from Milford Haven marina where salinity is below that of normal sea water. A local river flows into the marina, and the water mixes with sea water twice a day as the lock gates open to the sea. The salinity of

the marina can therefore vary. *Obelia longissima* was not found on rocks or sand as suggested by some authors (e.g. Hayward & Ryland 1994).

Obelia dichotoma was found growing on ropes, boards and rocks. It was also identified in rock pools and growing on the siphon of a tube worm and found at four different Pembrokeshire sites in total. These habitats reflect its assumed preference for inert substrata. Its season is different to *Obelia longissima* and *Obelia geniculata*. *Obelia dichotoma* arrived in late summer in Milford Haven marina, where it appeared to be tolerant of the varying salinity.

Obelia bidentata (bicuspidata)

Not yet found at sample sites in Pembrokeshire, but this may be due to the fact that it prefers warmer water. A new power station being built in the haven is predicted to raise water temperatures and therefore may result in this species colonising habitats in the Milford Haven waterway.

Photocyte patterns were successively recorded for three *Obelia* species and the luminous species *Clytia hemisphaerica*. The pattern was found to vary in each species, and identical results were obtained on samples from the Plymouth area as well as those in Pembrokeshire. The fluorescence of photocytes in the umbrella of the medusa (previously called *Obelia lucifera)* of *Obelia geniculata* was also recorded. These results build on the previous observations made by Jim Morin (Morin 1974).

The bioluminescence of the three *Obelia* species was recorded using the chemiluminometer. This confirmed their ability to produce a bioluminescent response, when compared to non-luminous, but taxonomically similar species such

as *Laomedea flexuosa*. The photoprotein obelin was successfully extracted from the 3 *Obelia* species. It rapidly produced light when tested with calcium ions in the chemiluminometer. The nature of the anion in the calcium solution was found to have no effect on this response as previously reported by other authors (Moisescu et al 1975).The Photek imaging system was also utilised to measure the rapid emission of light from obelin once calcium ions are added to it. A protocol was then developed to rapidly distinguish between the three species of Obelia. Photocyte patterns, chemiluminescence and photoprotein activity can all be rapidly tested in the laboratory to ensure the correct identification of the species. Furthermore luminous hydroids can be easily distinguished from non-luminous species using these methods. My hypothesis for the difference in position of photocytes is that it could due to *Obelia longissima* and *Obelia dichotoma* deterring a specific predator from eating their hydranths. This predator may be a nudibranch. Different species of nudibranch are known to eat different parts of the hydroid. Other predators have been observed on the colonies of *Obelia* at the MHPA jetty; these include amphipods and the crenoid *Ophiopholis aculeata*. These predators specifically consume the hydranths of *Obelia*. Some samples taken were completely denuded of hydranths.

For marine biologists, observing fluorescence could aid identification of the different species of *Obelia.* It also rapidly distinguishes *Obelia* species from the non luminous *Laemodea flexulosa* which in the past has often been misidentified as *Obelia*. It is a straight forward procedure to grow most species of hydroids in seawater tanks. However culturing *Obelia* species can be very problematic. In the

past scientists have cultured what they thought was *Obelia* only to discover that it was in fact *Laemodea* (Crowell, 1953).

Several examples of GFP in the base of the hydrothecae and tentacles of some *Obelia* specimens has been observed and recorded. Previously GFP was thought not to be in the hydrothecae. The FLIM (fluorescence life time imaging) data from this fluorescent observation was measured. This gave a half-life of 3 nanoseconds and this confirmed that it was due to the presence of GFP. It may be the case that previous research has been carried out on samples that were not that fresh and this may have a bearing on observations made by other authors. No bioluminescence was recorded in the hydranths.

Using this protocol to distinguish the 3 luminous species will ensure that the correct hydroids can be used for research and DNA analysis to finally establish if there are different species of *Obelia*.

3.4.2 Distinguishing between *Obelia* and taxonomically similar non luminous species of hydroids.

3.4.2.1 Distinguishing *Obelia geniculata* from *Laomedea flexuosa*

A consideration of habitat aids the identification process. *Laomedea flexuosa* is more lightly to be found in the middle shore on rocks or weeds (Hayward & Ryland, 1994). A hydroid specimen found on rock is unlikely to be *Obelia geniculata* as is any found above the mean tidal level, as it needs to remain submerged. Scientists working on thecate hydroids in the Russian White Sea have found that *Laomedea flexuosa* only occurs in the lower littoral zone, due to its

plannulae only swimming 30 minutes away from its hydroid before settling (Marfenin & Belorustseva, 2008). *Obelia geniculata* prefers moving water and is nearly always found on *Laminaria*. Marfenin and Belorustseva have found that *Obelia geniculata's* stem is less flexible than *Obelia longissima's* and that this is why it can withstand more current (Marfenin & Belorustseva, 2008). Examination under the microscope will show very similar taxonomic structure for both *Obelia geniculata* and *Laomedea flexuosa*. Measurement of bioluminescence utilising the chemiluminometer (refer to chapter two) will reveal the bioluminescent response of *Obelia geniculata,* whereas *Laomedea flexuosa* will not produce any bioluminescence. Observation under the fluorescent microscope will reveal the GFP dispersed throughout the uprights of *Obelia geniculata* but no GFP in *Laomedea flexuosa.* If any doubt remains an obelin extraction can be carried out. The luminous *Obelia geniculata* will contain obelin whereas the non luminous *Laomedea flexuosa* will not. This same protocol will distinguish the luminous *Obelia dichotoma* from the non luminous thecate hydroid *Hartlaubella gelatinosa*, which are again taxonomically very similar.

3.4.2.2 Distinguishing between the 4 species of *Obelia*.

Following the reports of Hastings and Morin that fluorescent patterns of two different *Obelia* species may vary the fluorescence pattern in specimens of three different species obtained in Pembrokeshire have been photographed. This has clearly shown that the photocyte dispersal is different in each species. *Obelia dichotoma's* photocytes are in the pedicle below the hydrothecae, but also in some areas of the upright. *Obelia geniculata's* photocytes are always dispersed in the

upright of the hydroid which agrees with the findings of Morin and Hastings. *Obelia longissima* has only one fluorescent photocyte positioned directly below the hydrothecae. The same fluorescent patterns were observed in specimens examined at Plymouth MBA and collected from the Plymouth area. These techniques also allow *Obelia* species to be accurately distinguished from taxonomically similar non bioluminescent hydroid species. In particular *Obelia geniculata* can be distinguished from *Laomedea flexuosa* and *Obelia dichotoma* from *Hartlaubella gelatinosa.* The same fluorescent patterns were observed for each *Obelia* species in specimens taken from both the Pembrokeshire and the Plymouth areas. The fluorescent pattern for the luminous hydroid *Clytia hemisphaerica* was also recorded as a comparison, and is again different from any of the *Obelia* species. I have also recorded several examples of GFP in the base of the hydrothecae and tentacles of some *Obelia* specimens. Previously GFP was thought not to be in the hydrothecae. The FLIM data from this fluorescent observation was measured. This gave a half-life of 3 nanoseconds and this confirmed that it was due to the presence of GFP.

It cannot be assumed that fluorescence observed in marine species is always due to GFP. At the Plymouth hydrozoan workshop in 2007 a siphonophore *Muggiaea atlantica* and 2 species of medusae appeared fluorescent under the microscope, but analysis using RGB ratios (refer to chapter 6) indicated this was not due to GFP. However this fluorescence due to other fluors can also be taxonomically valuable. Shin Kubota et al have since reported that both *Eugymnanthea japonica* and *Eugymnanthea inquilina* exhibit "auto fluorescence". The pattern of this auto fluorescence is different in the 2 species of the medusa (Kubota et al. 2008).

Future work

Fluorescent patterns of photocytes in other bioluminescent hydroids could now be studied as they may also prove useful in distinguishing between other species. The fluorescent pattern of the photocytes of medusae *Obelia lucifera* was recorded. In future what is thought to be the other species of *Obelia* medusa i.e. *Obelia nigra* fluorescent pattern could be studied to find if there are any differences.

Now that three species of Obelia can be accurately identified their full genome could be sequenced, so that it can finally be established whether they are separate species or different forms of one species. The c-DNA has been identified for the species but not the full genome. French scientists have recently sequenced *Clytia* and found it includes DNA which targets the mitochondria; therefore GFP may be a cell organelle.

FLIM has been utilised to ascertain that fluorescence observed in the hydranths of *Obelia dichotoma* was almost certainly due to GFP. Previously GFP was not thought to be located in hydranths. The hydranths do not produce bioluminescence therefore the role of GFP in the hydranths could now be investigated. It could be acting as a lure for prey or as UV protection. Alternatively it might be the site of GFP production. GFP has been recently identified in the snakelocks anemone, often found in shallow Pembrokeshire rock pools. It is likely that GFP in this non luminous organism is a lure for prey or a UV block. FLIM also indicated that 2 other fluorescent components may be present in the hydranth and this warrants further investigation. The hydranths may contain different coloured fluorescent proteins. Several research groups have attempted to clone the GFP in the hydroid stage of *Obelia geniculata,* but it proved elusive (Aglyamova et al. 2011).Morin

and Hastings observed obelin and GFP in the form of "sub-cellular granules" in *Obelia* but not in *Aequorea*." The structure of the granule prevented the externally added Ca^{2+} from accessing the photoprotein and triggering the luminescence" (Aglyamova et al. 2011: Morin & Hastings, 1971a).

Recently Aglyamova et al have successfully cloned fluorescent proteins in the medusa stage of *Obelia*. They discovered and cloned three distinct fluorescent proteins within the medusa. As well as the expected green fluorescent protein they isolated a yellow and cyan fluorescent protein. This is the first discovery of a range of coloured fluorescent proteins in Hydrozoa. Different coloured fluorescent proteins have already been found in several orders of the Anthozoa, including corals and sea anemones (Aglyamova et al. 2011).

Three of the 4 species of *Obelia* were found in the Milford Haven waterway. Specimens of these species were all found to be bioluminescent and as expected all contained the photoprotein obelin. The mean level of bioluminescence was found to be lower in samples of *Obelia longissima* taken from sample ropes in the Milford Haven marina. Considering the results for water quality at the sample sites, this does not appear to be due to any pollution in the marina. It might however be due to the lower salinity in the marina. Further investigations into the influence of salinity on bioluminescence should be undertaken. Due to global warming and the discharge of warm water in to the Milford Haven waterway from a new power station, the seawater temperature of the whole Milford Haven waterway may soon rise. This may herald the appearance of the fourth species of *Obelia bidentata* in the area. The fluorescent pattern of this species can then be studied. Alternatively specimens for study may be obtained from the USA. The

temperature of sea water at the Milford sample sites should be regularly monitored together with the viability of the Obelia colonies. If the temperature of the Haven does increase, this will form a model for the possible effects of global warming. Obelia should be able to withstand this as it is found in a wide variety of temperatures throughout the world. However if the warmer water increases red tides in the area, Obelia colonies may be detrimentally effected due to lack of zooplankton and oxygen.

Chapter four

How is light emitted in *Obelia*?

4. Introduction

4.1 How is light emission triggered in *Obelia geniculata?*

The most primitive animal nervous systems are exhibited by Cnidarians. Their nervous systems are termed nerve-nets, and consist of multi polar and bipolar cells forming a network beneath the epithelium layer. Long nerve cell processes run together creating small bundles of fibres (Passano & Passano, 1971). In *Obelia,* sensory cells within the ectoderm have nerve fibres, which run into the mesoglea forming part of a presumed nerve net. Titschack created an image of a nerve net by photographing silver impregnated sea pens. This revealed a nerve-net in the mesoglea of the sea pen with only a few connections to the nerve-nets in its ectoderm and endoderm (Titschack, 1964; Titschack, 1966; Morin, 1974). Whether *Obelia geniculata* itself possesses a nerve net has yet to be confirmed. Matthews concludes that the medusa of *Obelia* contains a nerve net, but the sessile hydroid does not. The existence of a nerve- net in the freshwater Cnidarian *Hydra* has been confirmed. "*Hydra*'s nerve net provides a more rapid spread of signals in a clear direction, whereas *Obelia* has electrical signals spreading non specifically through its epithelial sheet" (Matthews, 2001).

Mackie has demonstrated that conduction of signals can occur via epithelial tissue that does not contain nerves (Mackie, 1965). "Mackie showed nerve-free epithelia can conduct behaviourally meaningful signals" (Josephson, 1974). This demonstrates that the nerve-net is not the only conduction system in Cnidarians.

Cnidocytes which trigger the release of nematocysts are classed as a sensory cell and Cnidarians are thought to possess several other types of these sensory cells

which will respond to touch, chemicals, light or vibration (Josephson, 1974). The nervous systems of marine organisms are studied by evolutionary neurobiologists as they are thought to be examples of the first nervous systems to have evolved. Nerve nets in *Hydra* and sea anemones are considered to be living examples of early primitive nervous systems which lacked a central nervous system (Robson, 2011). Many of the most important studies of nerve nets have been carried out on sea anemones, *Hydra*, hydro medusae, siphonophores and scyphozoans rather than marine hydroids. Endodermal nerve cells have been reported in the fresh water *Hydra* (Davis, 1972). *Hydra's* structure is very similar to that of the marine thecate hydroids. The work of Josephson and Macklin has suggested that the inner and outer membranes of *Hydra* may be independently excitable. Josephson and Macklin have also demonstrated that calcium ions move from the intercellular space of *Hydra* into its ectodermal epithelio muscular cells (Josephson & Macklin, 1969).

In 1968 Chapman observed bipolar cell bodies in *Obelia* medusae (Chapman, 1968). Non-nervous epithelial conduction has been shown to occur in the sub umbrella, ectoderm and endoderm of some hydromedusae (Mackie and Passano, 1968).Medusae have to swim and therefore have a more complex nervous system than that of the sessile hydroid. They have a double nerve net and nerve rings. The sub umbrella's muscles are controlled by an inner nerve ring, whereas an outer nerve ring relays impulses received from statocysts. Statocysts are cells which sense any change in orientation of the medusa (Vines & Rees, 1968). Non-nervous epithelial conduction has also been demonstrated in the stolons of the hydroid *Cordylophora*.

"Conducting systems in hydrozoans have been shown to produce large electrical signals of several millivolts" (Josephson, 1974). The minute fibres of a nerve-net are unlikely to be able to produce such a large signal (Morin & Cooke, 1971). Therefore the signal must be due to a non nervous epithelial conduction system. However nerve activity may trigger these electrical signals and the findings of Ball and Case support this scenario. They conducted experiments on the hydroid *Corymorpha palma* (Ball & Case, 1973). In stolons and uprights of *Obelia* the sites requiring facilitation are separated by 1.3 mm (Morin and Cook, 1971c). Facilitation is when a previous sub threshold stimulus leaves an effect which adds to a second stimulus to trigger a response.

Assuming there is electrical transmission between epithelial cells, a signal could originate at one site and then travel across to adjacent cells in turn, exciting them. Chemical synapses have not been found between epithelial cells therefore any such signal must travel as a result of an induced depolarisation of the hydroids membrane (Josephson, 1974).

Non-nervous epithelial conduction has been recorded in the following hydroids: *Hydra (*McCullogh, 1965), *Tubularia* (Josephson & Mackie, 1965), *Cordylophora (*Mackie, 1968) and in *Obelia geniculata* (Morin & Cooke, 1971). In all cases these were large epithelial signals which were also relatively long lasting (200 millisec). Morin and Cooke state how these epithelial signals are "extensively distributed within a hydranth "of *Obelia geniculata.* They also note that non-nervous large slow potentials are only recorded in hydrozoans. Non-nervous potentials in Scyphozoa are smaller and faster (Morin & Cooke, 1971). Morin and Cooke also discovered that these large slow potentials were associated with 3

distinct movements in *Obelia geniculata.* The movements are all thought to be behavioural responses.

(i) Hydranth contraction

(ii) Mouth opening

(iii) Flexing of an individual tentacles.

Morin and Cook measured the electrical activity associated with these movements using a fine-tipped suction electrode. The electrode was connected to a tentacle or the hypostome of *Obelia.* The origin of these electrical potentials is still not known. They also established that there is no communication between the hydranths in *Obelia*. Each of the hydranths responds individually to a stimulus (Morin & Cooke, 1971; Morin, 1974).

Mackie & Passano's 1968 research on hydromedusae suggests that an epithelial system and primitive nerve net may interact. More complex responses are transmitted by the nerve net, whereas the epithelial system transmits messages for simple responses. In 1967 Dtrehler and Brandes whilst working on *Campanularia flexuosa,* studied the ultra structure of the hydranths. They found little evidence of any neural fibres within the hydranths. If the fibres did exist, it would be a challenging task to locate them as the hydranths are relatively small (Morin & Cooke, 1971).

 The exact mechanism, by which calcium ions enter photocytes triggering bioluminescent emission from obelin, has yet to be elucidated. A wave–like spread of bioluminescence is observed in *Obelia*, when it is stimulated with potassium

ions. Hastings and Morin described the bioluminescence of *Obelia geniculata* "a conducted scintillating emission emanates as a wave along the colony from individual photocytes. Repetitive waves may occur from a single stimulus" (Hasting & Morin, 1998). A mere diffusion of calcium ions through gap junctions alone is unlikely to produce such a response. Dunlap et al investigated the mechanism of bioluminescence in the photocytes of *Obelia* in 1987. They found that if extra cellular calcium ions are removed no light emission is observed. This suggests that voltage gated channels exist in the cell membrane of the photocyte cell, which control calcium ion entry. Dunlap isolated the photocytes but could not obtain any readings from them to prove that this suggested mechanism was functioning. However a "voltage-dependent calcium current was recorded by the group from non-luminous support cells" close to the photocytes (Dunlap et al. 1987). They also discovered that adding octanol or heptanol to support cells blocked the bioluminescence. Octanol and heptanol are both short chain alcohols which are known to block gap junctions (Brehm et al. 1989). They utilised voltage-clamp analysis and depolarised the support cell membranes using potassium ions. This has led to the hypothesis that calcium ions enter support cells producing chemical signals, which cross intercellular gap junctions, triggering light emission. Similar gap junctions are already known to exist in cardiac muscle. In 1980 Anderson demonstrated that action potentials can pass through gap junctions in coelenterates (Anderson, 1980).

The chemical signals are most likely calcium ions themselves, which can rapidly react with obelin. *In vivo* they trigger light emission from obelin in 1ms. Obelin is assumed to be in the cytoplasm of the photocyte rather than being bound in a

separate structure. These results do not totally discount the alternative theory that a different chemical signal crosses gap junctions, stimulating calcium ion release from an intercellular store. Dunlap also conducted experiments on isolated cell pairs of support cells and photocytes. However the speed of the bioluminescent response from these was far slower than the normal in *vivo* rate constant for the reaction.

Further experiments by Brehm et al have led to the hypothesis that in addition to gap junctions between support cells and photocytes, there are "voltage independent calcium permeability mechanisms" in the photocyte plasma membrane. They found that some areas of the photocyte do not luminesce despite the fact they contain obelin. This suggests that calcium ion diffusion is restricted and not via evenly distributed calcium channels (Brehm et al, 1989).

The bioluminescence of *Obelia geniculata* has also been studied to research the relationship between intracellular calcium and facilitation. This is in order to discover the mechanism of facilitation at neural synapses. Naranjo et al have conducted experiments using suction electrodes to link apparent facilitation in the bioluminescent flashes of *Obelia,* to the binding of three calcium ions to each molecule of obelin. They electrically stimulated hydroids with a frequency of between 0.1 to 1Hz. They hypothesised that facilitation occurs because obelin can have one or two calcium ions bound to it for some time without emitting light. It is only when the third calcium ion binds that bioluminescence is triggered (Naranjo et al. 1994). This agrees with a calcium hypothesis proposed in 1968 by Katz and Miledi (Katz & Miledi, 1968).

Mackie and Meech have investigated the action potentials which allow *Aglantha digitale* hydromedusae to swim. They found that it had two different types of movement "fast swimming" to escape from predators, and "slow swimming" which it used when capturing prey. Fast swimming resulted from "Na^+ dependent action potentials", but the slow swimming depends on a low amplitude calcium spike. Calcium ion channels in the axon produce this spike. This illustrates that the eight giant motor axons in this medusa can carry two different types of impulse (Mackie & Meech, 1985: Meech, 2004).

The aim of this chapter was to investigate how light emission is triggered in *Obelia* species.

The experimental strategy involved investigations into:

The effects of a range of potassium ion concentrations, on the bioluminescence of *Obelia* using the chemiluminometer.

Whether channel blockers would stimulate the hydroid membrane to produce a bioluminescent response, utilising the chemiluminometer.

The total light image of bioluminescence from different species of *Obelia* recorded using the Photek imaging system. Images were analysed using OAS and matrices to gain more information on patterns of photocyte emission.

4.2 Methods

4.2.1 The effect of potassium ion concentration on bioluminescence.

Individual hydroids from the 3 *Obelia* species were stimulated by potassium ions in the chemiluminometer as previously described in chapter 2. A range of potassium ion concentrations were prepared so that they were isotonic to a 0.5 M KCl solution e.g. a 0.25 M KCl solution also contained 0.25 M NaCl. Six hydroids of each species were stimulated with each concentration of potassium ions and the mean response calculated.

4.2.2 The effect of ion channel blockers on the bioluminescence of *Obelia* species.

A similar method to that of 4.2.1 was used. 1 ml of an ion channel blocker solution was injected onto the hydroid in the LP3 tube to ascertain if it would stimulate a bioluminescent response.

4.2.3 Calculating odd area set data (OAS)

Photek analysis software was utilised throughout to measure the number of photons being emitted from each photocyte and the pattern of that emission. Ten odd area set (OAS) circles are drawn on the brightest part of the total image from the specimen. These areas appear red on the pseudo coloured total light image. The software then calculates the photons produced by each OAS (i.e. a hot area). An Excel spread sheet was then used to graph the emission of each individual OAS area.

4.3 Results chapter four

The relative mean bioluminescence of 3 different species of Obelia was measured using the chemiluminometer refer to Fig 4.1.

Comparative bioluminescence of *Obelia* species
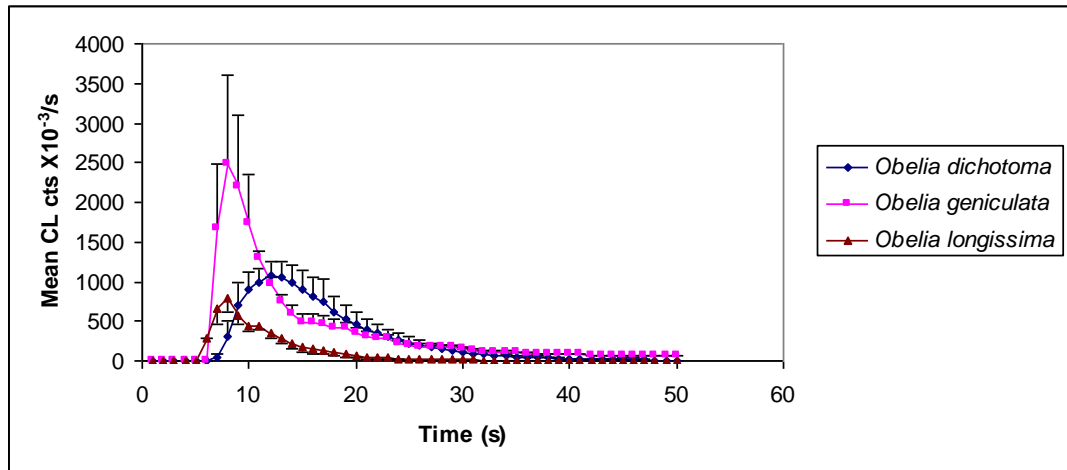
Fig.4.1. Comparative bioluminescence of *Obelia* species. Bioluminescence of the 3 species of *Obelia*. Bioluminescence of *Obelia* was stimulated by 1ml 0.5M KCl. Results represent the mean of six individual hydroids ±SEM.

When the bioluminescence of each of the individual six hydroids for each species is plotted, it was seen, that despite the fact they are all bioluminescent, the degree of bioluminescence in hydroids from the same colony shows considerable variation. *Obelia dichotoma* appears to have a broader but slightly lower mean bioluminescent response compared to *Obelia geniculata*. However its mean bioluminescence is greater than that of the colony of *Obelia longissima.*

Fig.4.2. The bioluminescence of six *Obelia* hydroids

(a) Bioluminescence of 6 *Obelia geniculata* hydroids

(b) Bioluminescence of 6 *Obelia dichotoma* hydroids

(c) Bioluminescence of 6 *Obelia longissima* hydroids
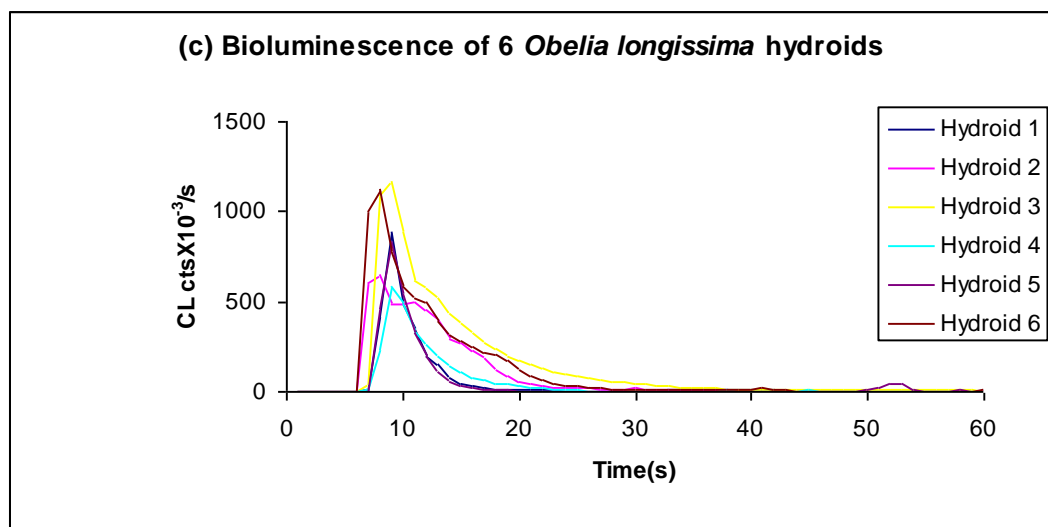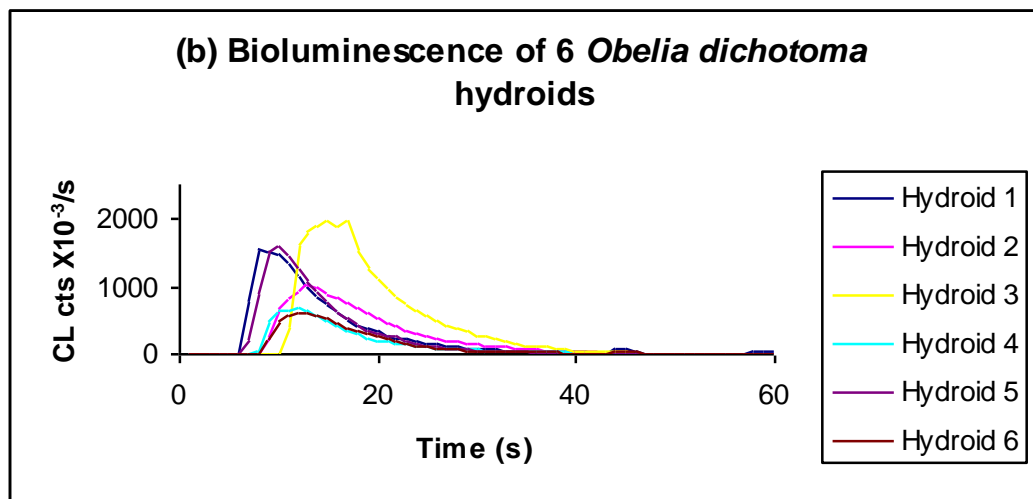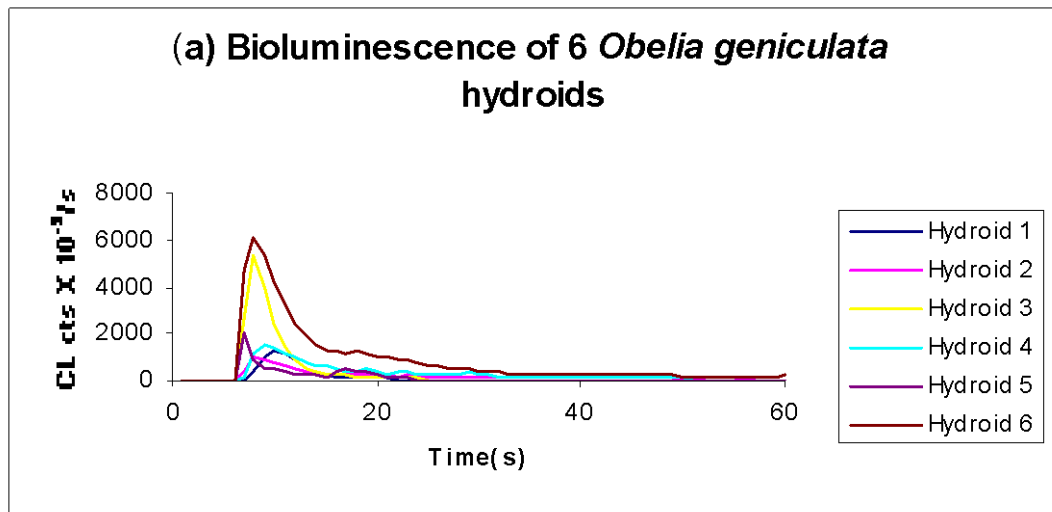
In all three species the addition of 1ml 0.5M KCl produced a bioluminescent response, but the amount of light produced did vary considerably.

Comparing the mean bioluminescence in Fig.4.1. showed that *Obelia geniculata* from the MHPA jetty showed a higher mean bioluminescence than *Obelia dichotoma* or *Obelia longissima*. *Obelia longissima* has less photocytes per hydroid than the other two species; therefore the total light produced by each species was divided by the mean number of functioning hydranths on each specimen hydroid. This "normalisation" gave a more accurate comparison of the degree of bioluminescence (refer to Fig.4.3.). This again showed that *Obelia geniculata* produced more light per hydranth than the other two species.

Mean chemiluminometer counts/minute from *Obelia* species
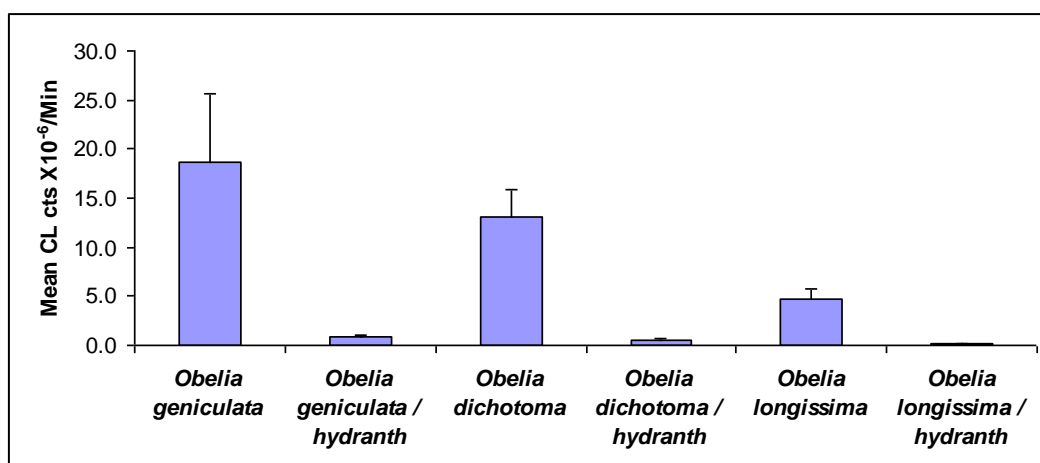
Fig.4.3. **Mean chemiluminometer counts from *Obelia* species/Min.** This shows a comparison of the total light emitted per minute from each *Obelia* species. Results represent the mean of six individual readings ± SEM. Results are also presented as light emitted per hydranth, per minute.

T test for bioluminescence of different species of *Obelia*

Species	P value	% similarity
Obelia geniculata against *Obelia longissima*	0.002	0.2
Obelia geniculata against *Obelia dichotoma*	0.013	1.3
Obelia dichotoma against *Obelia longissima*	0.003	0.3

Table.4.1. A T-test for the bioluminescence of different species of *Obelia*. The bioluminescence of *Obelia geniculata* was highly significantly different to that of *Obelia longissima.* The bioluminescence of *Obelia dichotoma* was also highly significantly different to that of *Obelia longissima.* The percentage similarity between the bioluminescence of *Obelia geniculata* and *Obelia dichotoma* was more similar, but still significantly different.

Lower bioluminescence of *Obelia longissima* may be due to the fact that it has less photocytes per hydroid, or the lower salinity which the specimen of *Obelia longissima* lives in may influence the degree of bioluminescence. This effect is worthy of further investigation. The different species were obtained from different habitats ie *Obelia geniculata* from *Laminaria* on the port authority jetty, *Obelia longissima* from ropes in Milford Haven marina and *Obelia dichotoma* from the siphon of a tube worm at Newgale. The differences in bioluminescence could be due to these differences in habitat.

Effect of potassium on the bioluminescence of *Obelia geniculata*
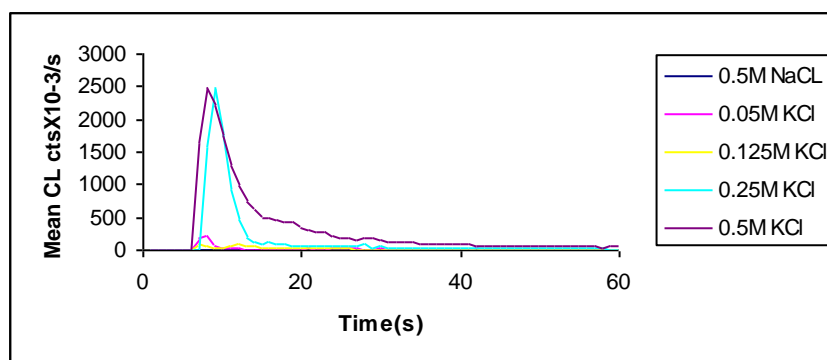
Fig.4.4. Effect of potassium on bioluminescence of *Obelia geniculata*. Results represent the mean of six individual hydroids.

Halving the concentration of potassium ions used to trigger bioluminescence, produced a sharper response, but interestingly concentrations below 0.25 M were insufficient to produce significant depolarisation of the hydroid membrane. This concentration of K^+ would easily depolarise a human nerve. Importantly the control 0.5 M NaCl produced no response above the background. This confirmed that the correct concentration of K^+ needed to elicit a bioluminescent response

from Obelia geniculata is 0.5M. It also suggests that K^+ does not easily pass through the outer perisarc of the hydroid.

Effect of potassium on bioluminescence of *Obelia dichotoma*
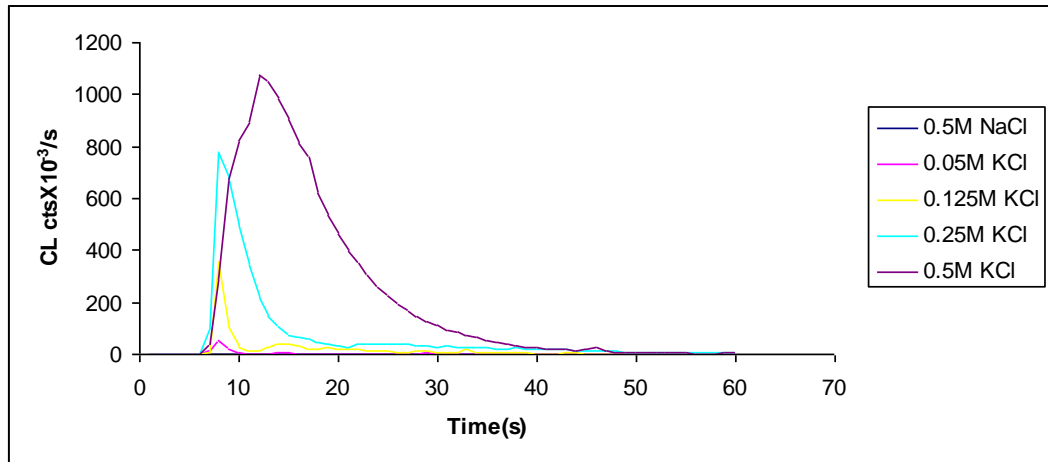
Fig.4.5. Effect of potassium on bioluminescence of *Obelia dichotoma*. Results represent the mean of six individual hydroids. A far larger and broader response is achieved when 0.5M KCl is used to trigger the hydroid's bioluminescence.

Effect of potassium on bioluminescence of *Obelia longissima*
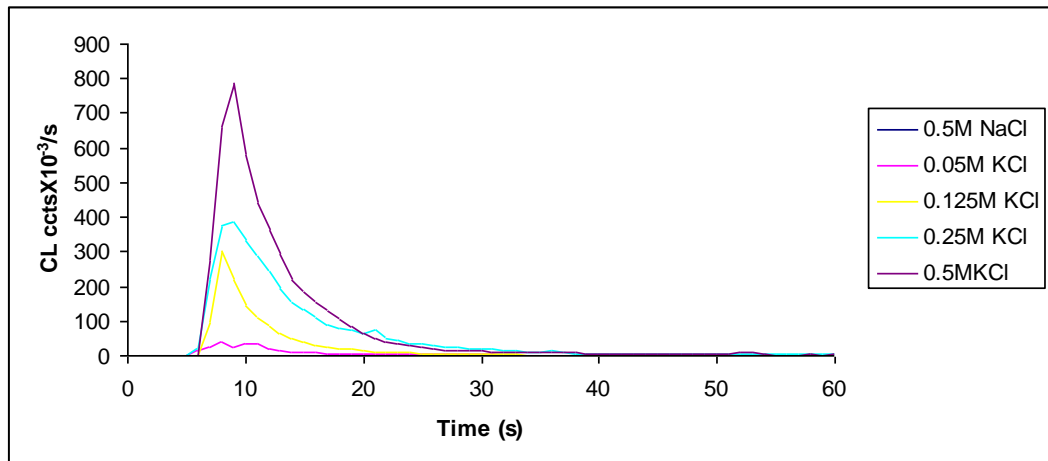
Fig.4.6. Effect of potassium on bioluminescence of *Obelia longissima*. Results represent the mean of six individual hydroids.

The effect of potassium on the overall bioluminescence of *Obelia*
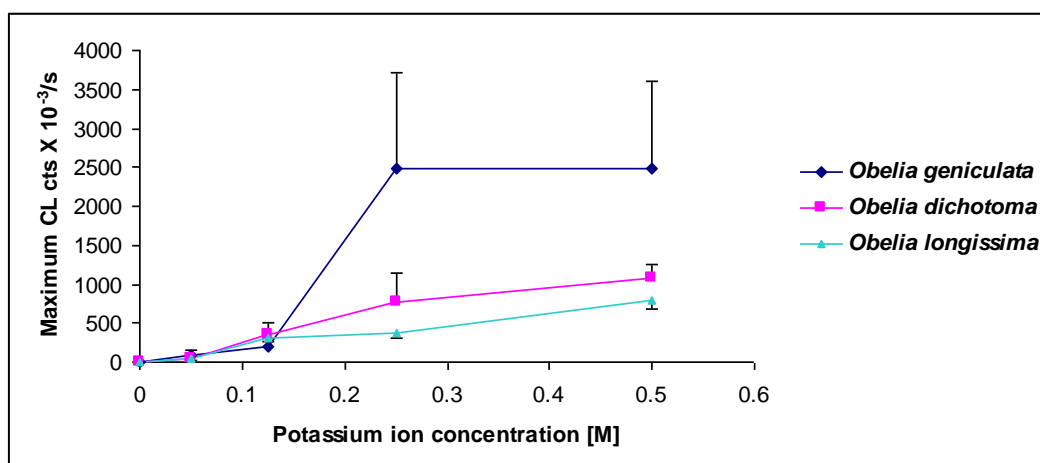
Fig.4.7.The effect of potassium on the overall bioluminescence of *Obelia*. A Summary of the effect of potassium ion concentration on bioluminescence of three species of *Obelia*. Results represent mean maximum chemiluminometer counts/s verses potassium ion concentration. Results represent the mean of six individual hydroids ±SEM. SEM was high at 0.25 and 0.5M for *Obelia geniculata*

4.3.3 Recording bioluminescence using the Photek imaging system.

Figs.4.8. and Fig.4.9. illustrate that the bioluminescence produced from *Obelia geniculata* is rapidly triggered by adding 1 ml 0.5 M KCl and that it is emitted from photocytes in the coenosarc rather than in the feeding hydranths or reproductive gonothecae. The position of photocytes appears in red on the pseudo colour palette.
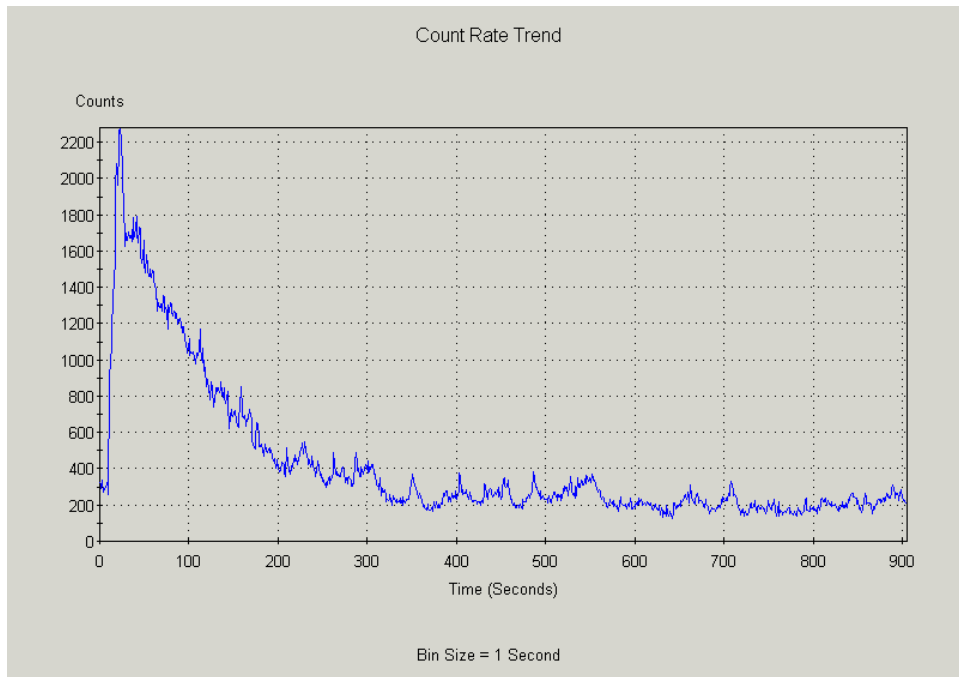
Fig.4.8. Bioluminescent response from 1 *Obelia geniculata* hydroid after injection of 1 ml of 0.5 M KCl. Injection at 5 seconds, triggered light production for over 60 seconds.
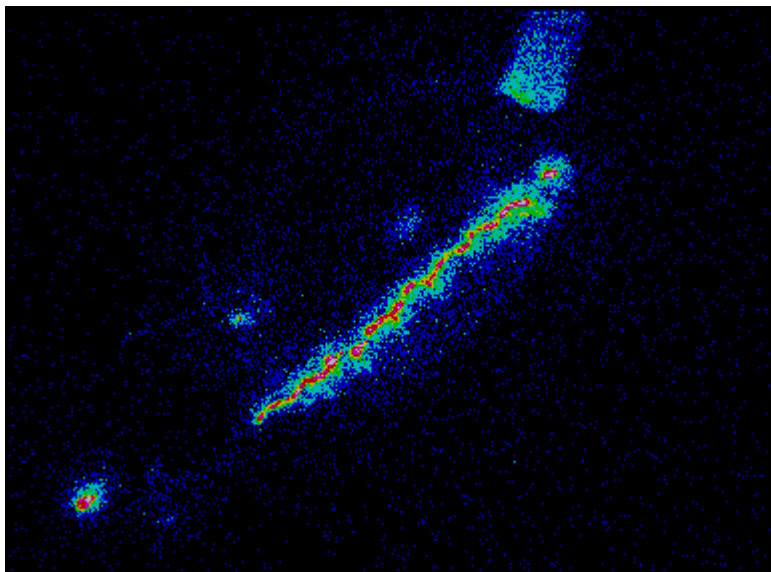
Fig.4.9. Total light image from 1 *Obelia geniculata* hydroid. Hydroid size 1.6cm pseudo colour image recorded with Photek imaging camera. Bioluminescence stimulated by injection of 0.5 M KCl, at 5 seconds. Red= bright light being emitted. Blue=dim light. Black=no light.

133

Fig.4.10. Bright field image from one *Obelia geniculata* hydroid. Before injection of 1 ml of 0.5 M KCl.
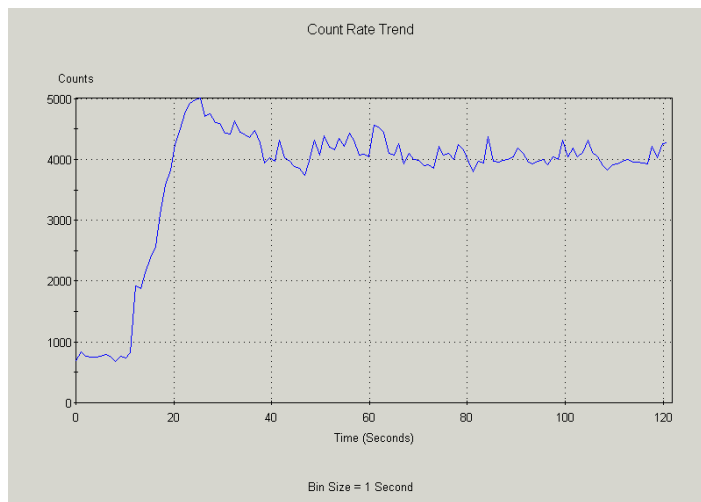
Fig.4.11. Bioluminescent response from 5 *Obelia geniculata* hydroids after injection of 1 ml of 0.5 M KCl. Injection at 10 seconds. As expected, this produced far more light than that emitted from one hydroid. Again the light is concentrated in the coenosarc of the hydroids where photocytes are found. The response continued for over 2 minutes.
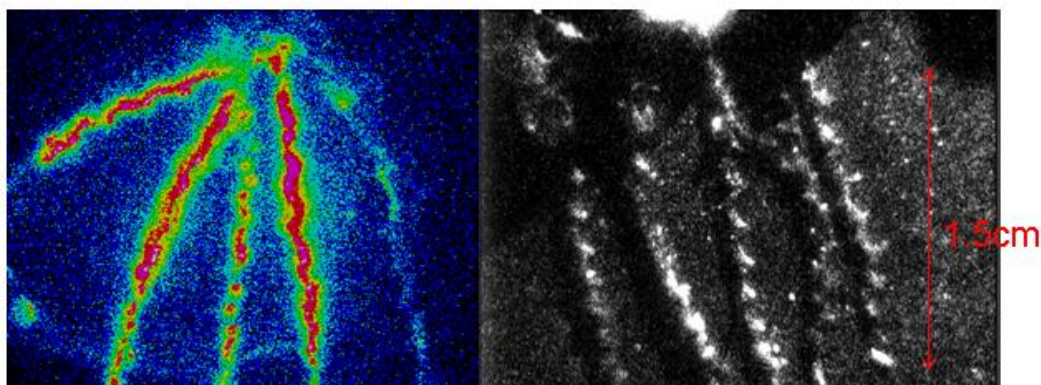
Fig.4.12. Left hand picture shows the total light image from 5 *Obelia geniculata* hydroids after injection of 1ml of 0.5 M KCl. Injection of 0.5M KCl at 5 seconds. The right hand picture shows the bright field image. Each hydroid was approximately 1.5cm long.

Bioluminescent emission from photocytes
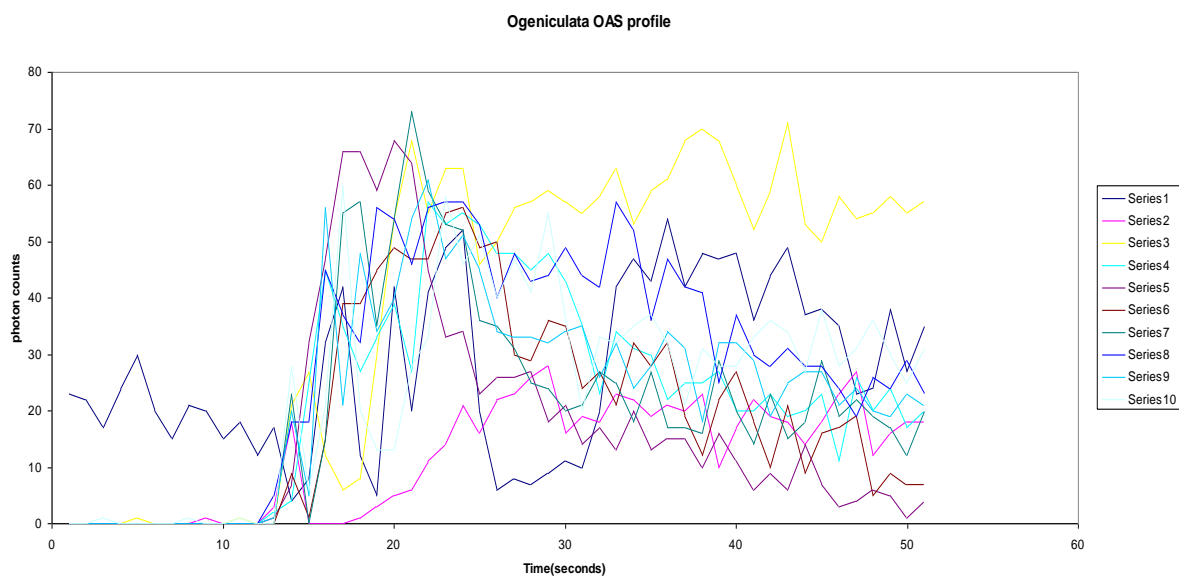
Fig.4.13.Ten Odd Area Sets from *Obelia geniculata*. This illustrates the photons emitted from ten hot areas from one *Obelia geniculata* hydroid. The "hot areas" corresponded to the positions of photocytes.

135
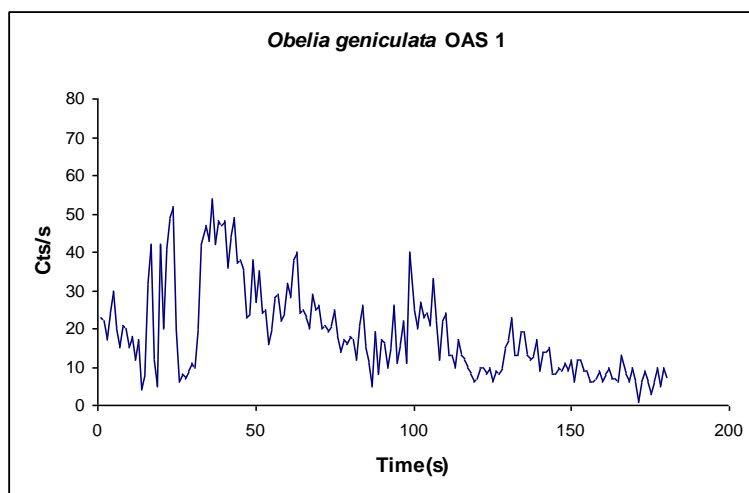
Fig.4.14. One Odd Area Set of one hot area from one *Obelia geniculata* hydroid. This showed multiple peaks of light emission lasting over 60 seconds, being emitted from one photocyte.

Figs.4.15. (2 to 10). Individual Odd Area Set photon counts from *Obelia geniculata*. These all show evidence of oscillation of light and that light emission lasts over two minutes.

136

Obelia geniculata OAS 4

Obelia geniculata OAS 5

Obelia geniculata OAS 6

Obelia geniculata OAS 7

Obelia geniculata OAS 8

Obelia geniculata OAS 9

Obelia geniculata OAS10
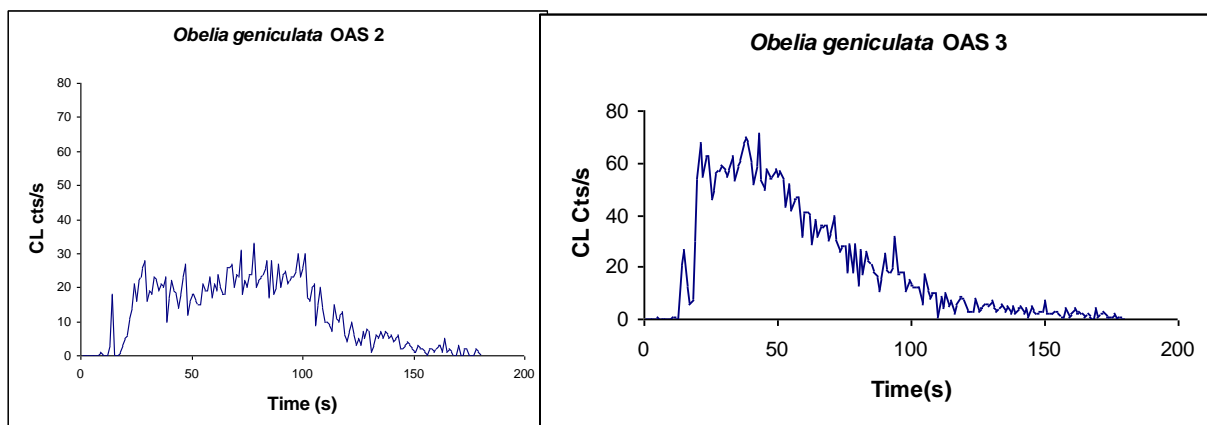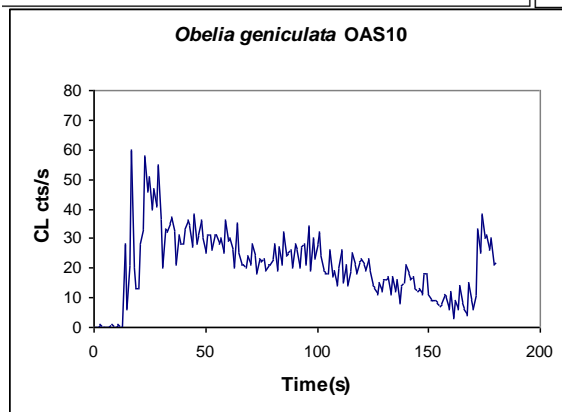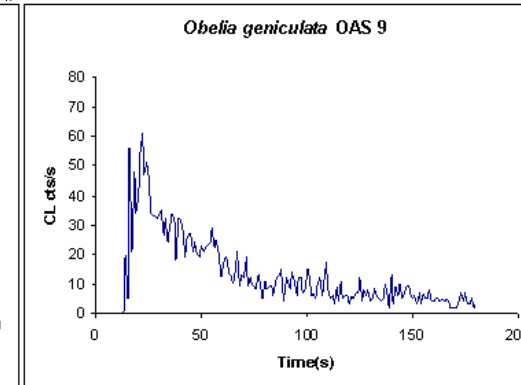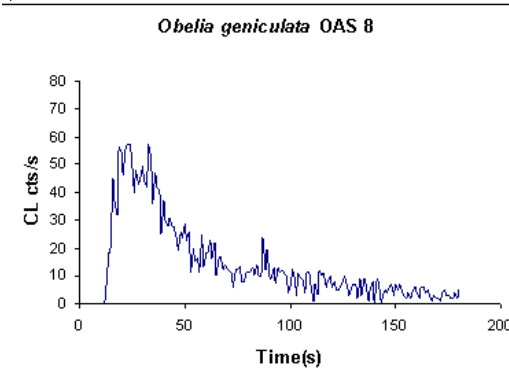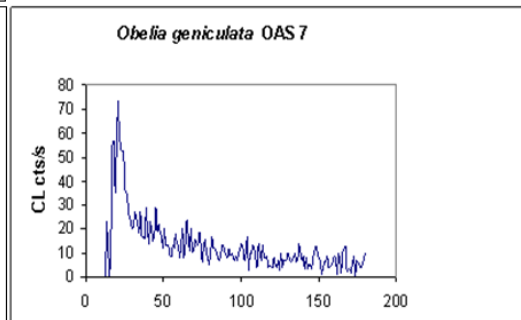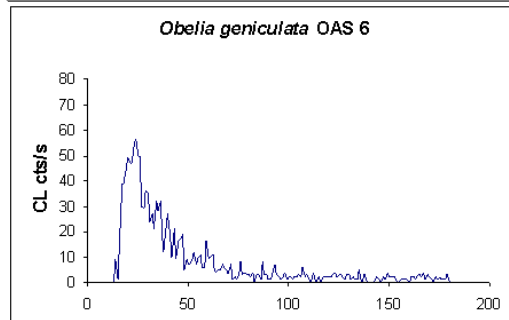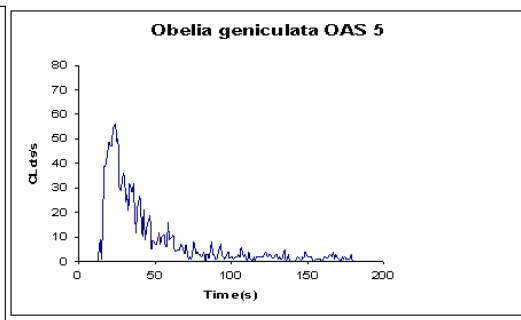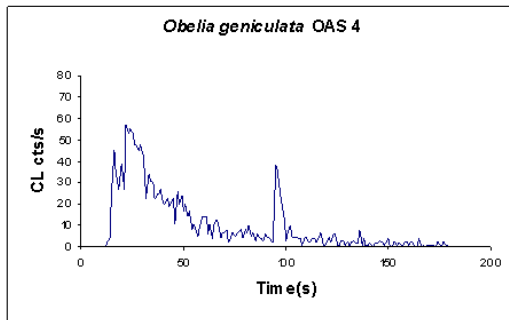
Fig.4.16.Ten Odd Area Sets for 10 hot areas from one *Obelia longissima* hydroid.

Olong5 extracted OAS

Ten Odd Area Sets for 10 hot areas from one *Obelia longissima* hydroid.
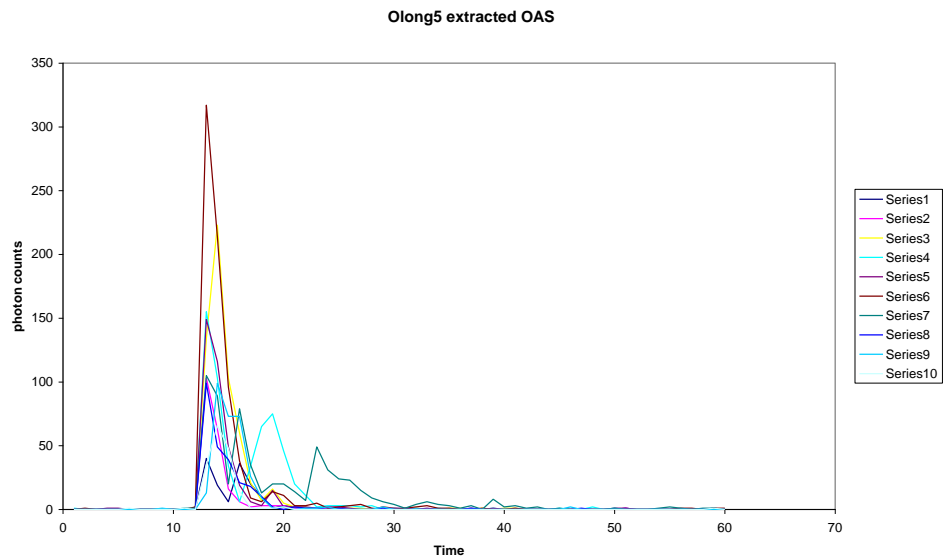
Obelia longissima OAS 1
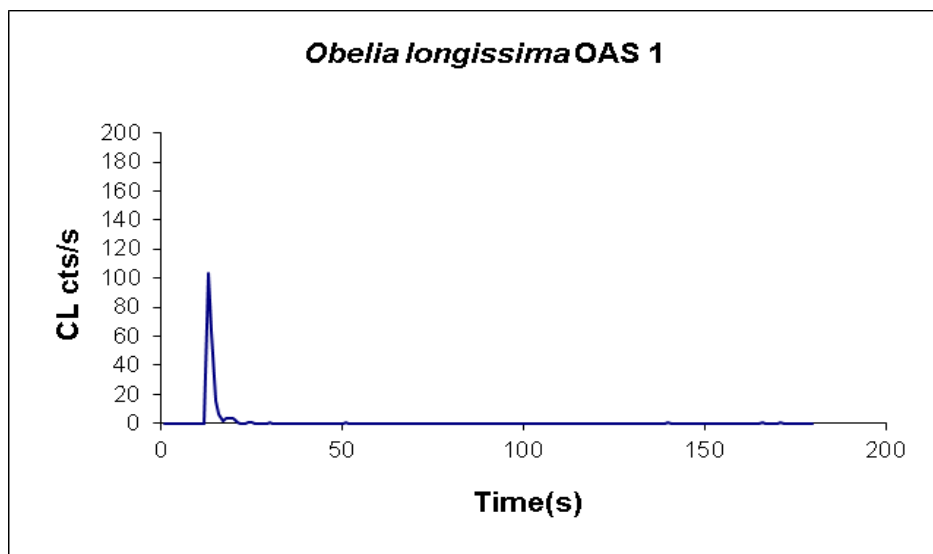
Fig.4.17. One Odd Area Set from *Obelia longissima.* The pattern of light emission is different to that of *Obelia geniculate*. Emission is a sharp peak lasting less than 30 seconds.

138

Fig.4.18.Odd Area Sets 2 to 10 for Obelia longissima.
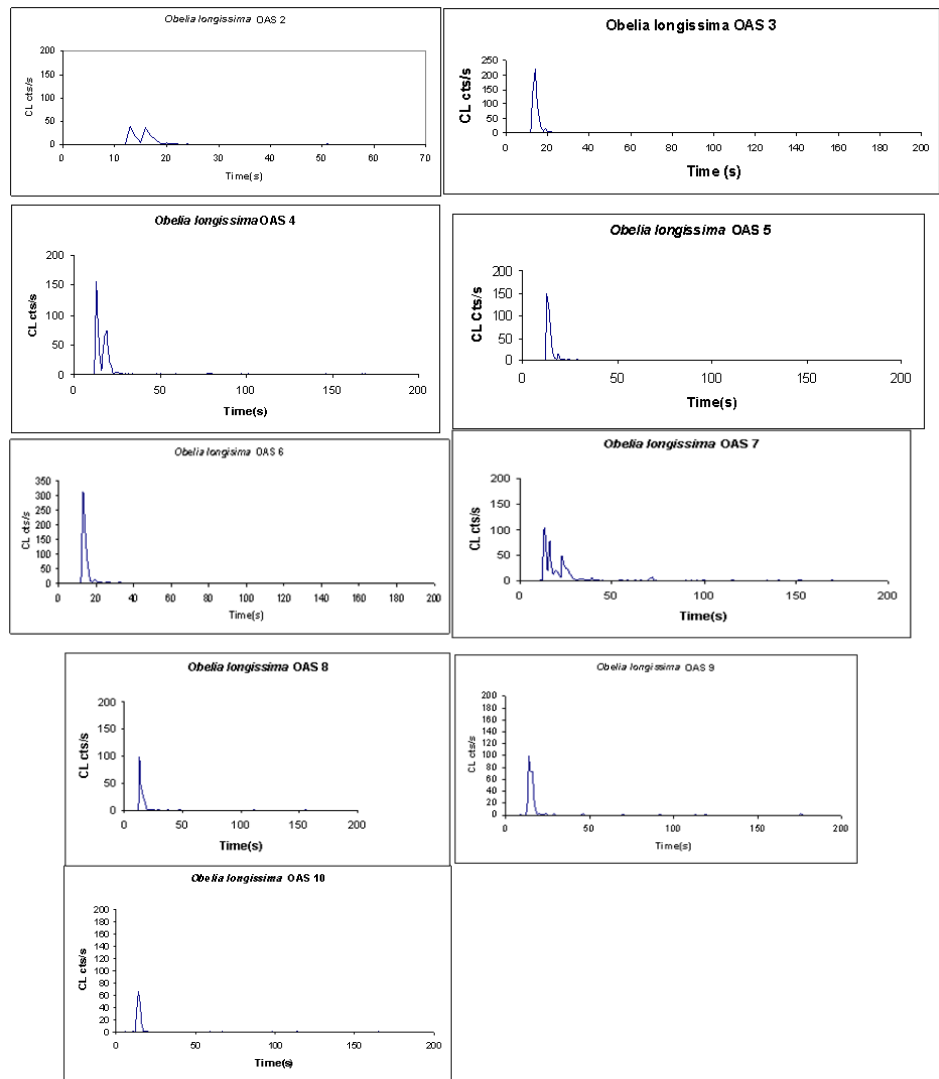
Odd Area Set 2. 1 to 10 for Obelia longissima. All emissions were shorter and had less intensity than those for *Obelia geniculata*.
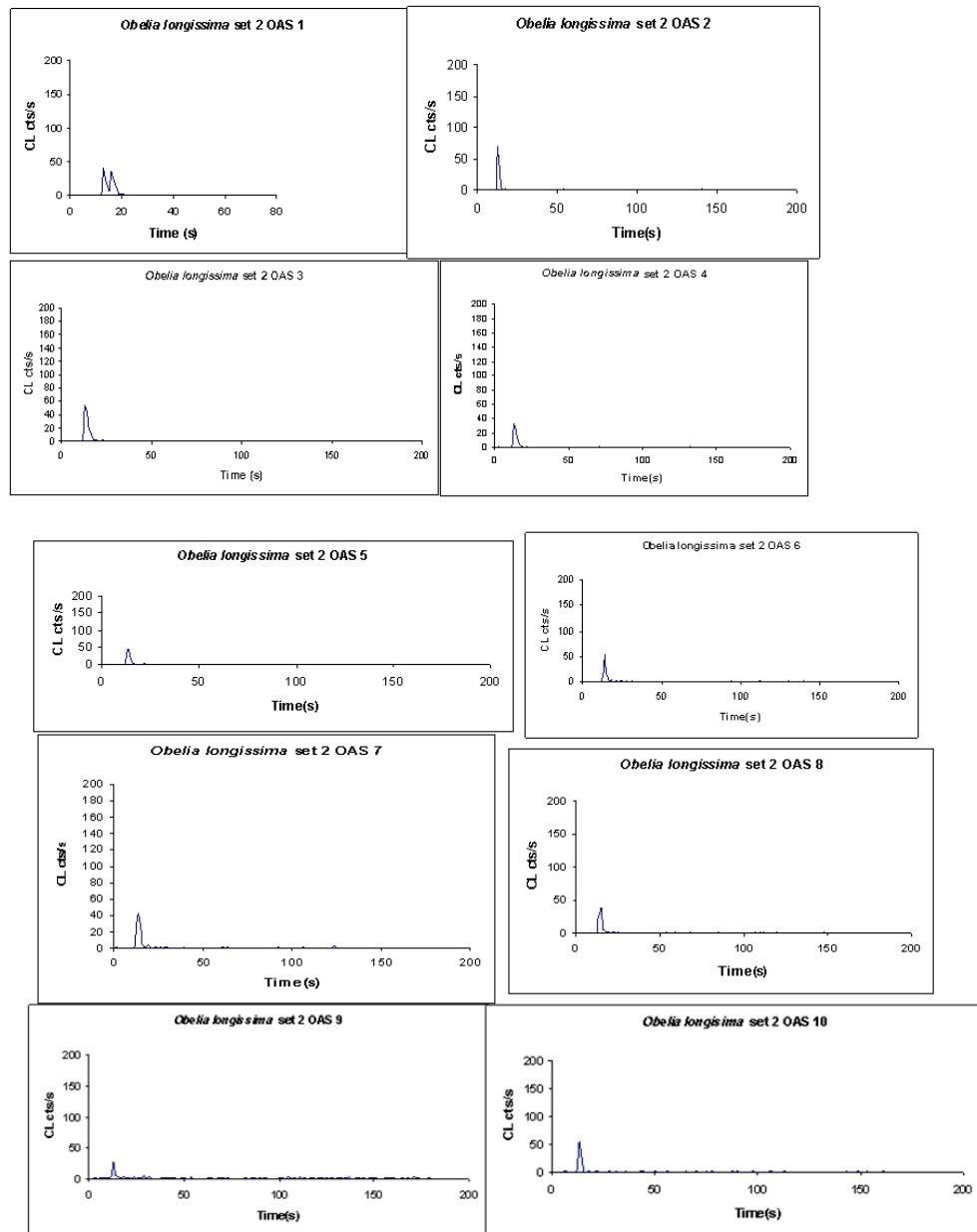
Fig.4.19.Odd Area Set 2. One to ten for Obelia longissima. All emissions were shorter and had less intensity than those for *Obelia geniculata.*

4.3.4 The effect of ion channel blockers on bioluminescence
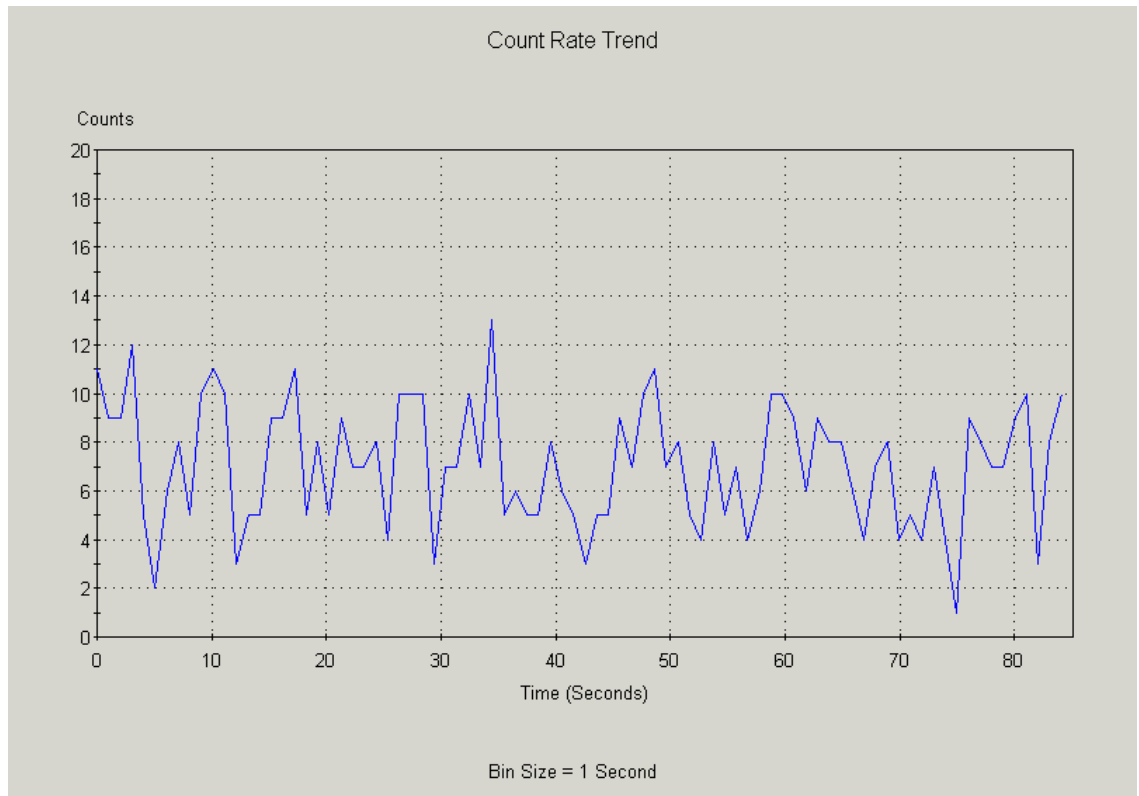
Fig.4.20. The effect of octanol on the potassium stimulated bioluminescence of *Obelia geniculata.* Hydroid soaked for 30 min in 10 mM octanol prior to injection of 1 ml of 0.5 M KCl. Recorded using Photek imaging system.

Octanol severely reduced the bioluminescent response of *Obelia geniculata*. It is known to block gap junctions, so this result supports the hypothesis of Brehm et al that gap junctions exist between the support cells and photocytes in Obelia. However the outer membrane of the hydroid may be disrupted by the octanol so this effect requires further investigation.

Fig.4.21.The effect of tetraethylammonium chloride(TEA) on *Obelia geniculata*
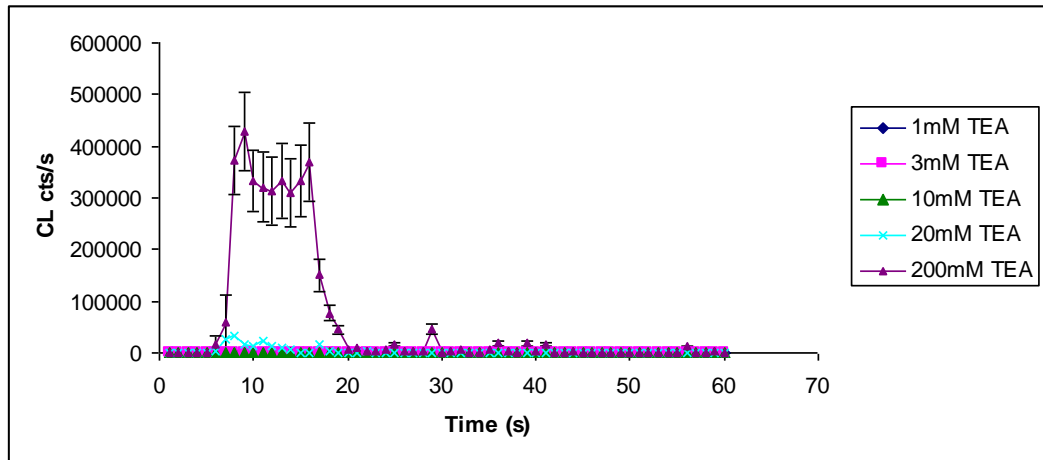
The effect of tetraethylammonium chloride(TEA) on *Obelia geniculata*. The effect of tetraethylammonium chloride (TEA) at a range of concentrations on the bioluminescent response of *Obelia geniculata.* 1ml of TEA at a concentration of 200mM triggered a bioluminescent response from *Obelia geniculata*, but this produced far less photons than the same volume of 0.5M KCl. Lower concentrations of this blocker did not elicit a meaningful response. Results represent the mean of six individual hydroids ±SEM. TEA may be blocking potassium ion channels in the hydroid or it might produce an irritation response. TEA is known to block potassium ion channels.

Fig.4.22. The effect of tetraethylammonium chloride (TEA) on the overall bioluminescence of Obelia *geniculata.*
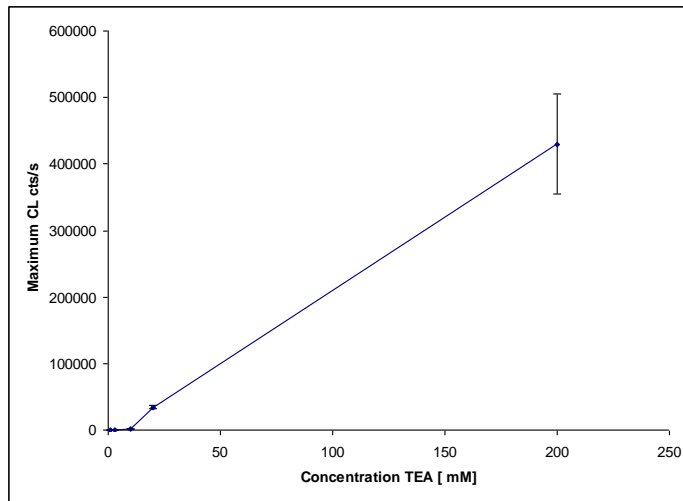
The effect of tetraethylammonium chloride (TEA) on the overall bioluminescence of *Obelia geniculata.* A summary of the effect of tetraethylammoniumchloride (TEA), a potassium ion channel blocker on the bioluminescence of *Obelia geniculata.* Only the relatively high concentration of 200 mM produced a significant photon response.

Figs 4.23 and 4.24 illustrate the similar but not identical response was seen when

Obelia dichotoma and *Obelia longissima* were simulated with TEA. 200 mM TEA

only produced a very small response in *Obelia longissima.* Further experiments

need to be conducted in order to investigate this difference between the species.

Fig.4.23. The effect of tetraethylammonium chloride (TEA) on *Obelia dichotoma*
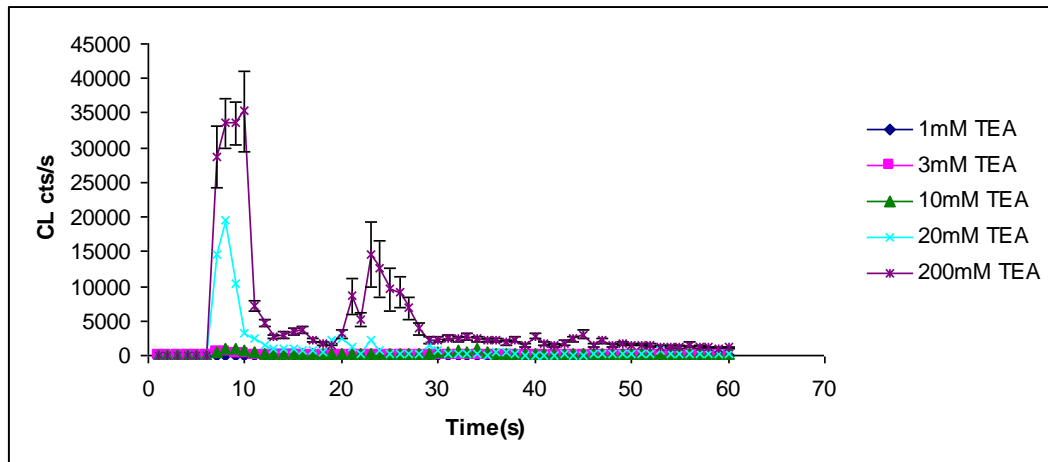
 The effect of tetraethylammonium chloride on *Obelia dichotoma.* A summary of the effect of Tetraethylammoniumchloride, a potassium ion channel blocker, on the bioluminescence of *Obelia dichotoma.* Results represent the mean of six individual hydroids ±SEM.

Fig.4.24.The effect of tetraethylammonium chloride on *Obelia longissima*
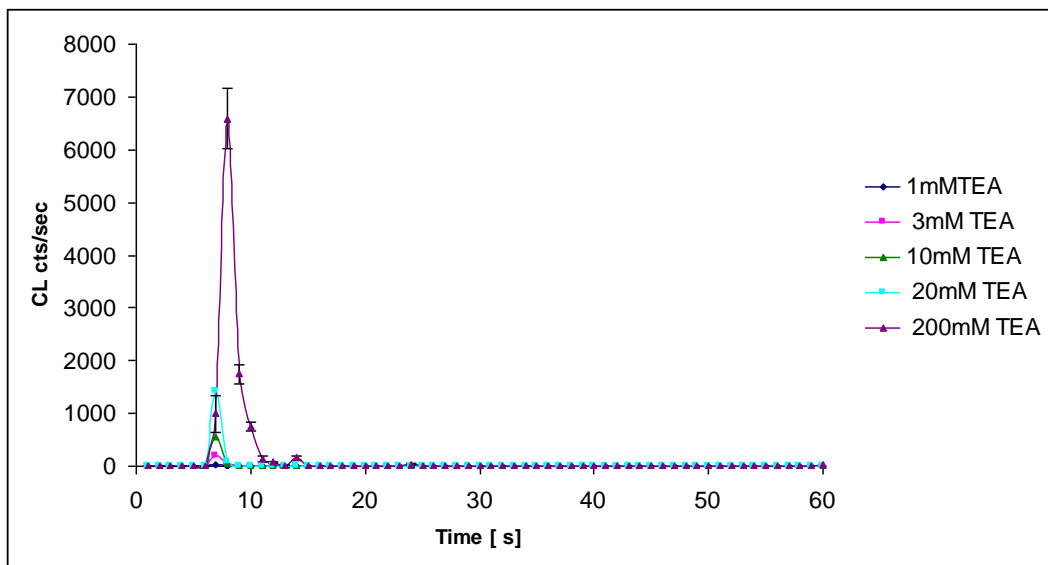
The effect of tetraethylammonium chloride on *Obelia longissima.*
 A summary of the effect of Tetraethylammoniumchloride (TEA), a potassium ion channel blocker on the bioluminescence of *Obelia longissima.* Results represent the mean of six individual hydroids ±SEM. 200 mM TEA produced a much smaller response in this species of *Obelia.*

Fig.4.25. The effect of 4-aminopyridine [4-AMP] on *Obelia geniculata*
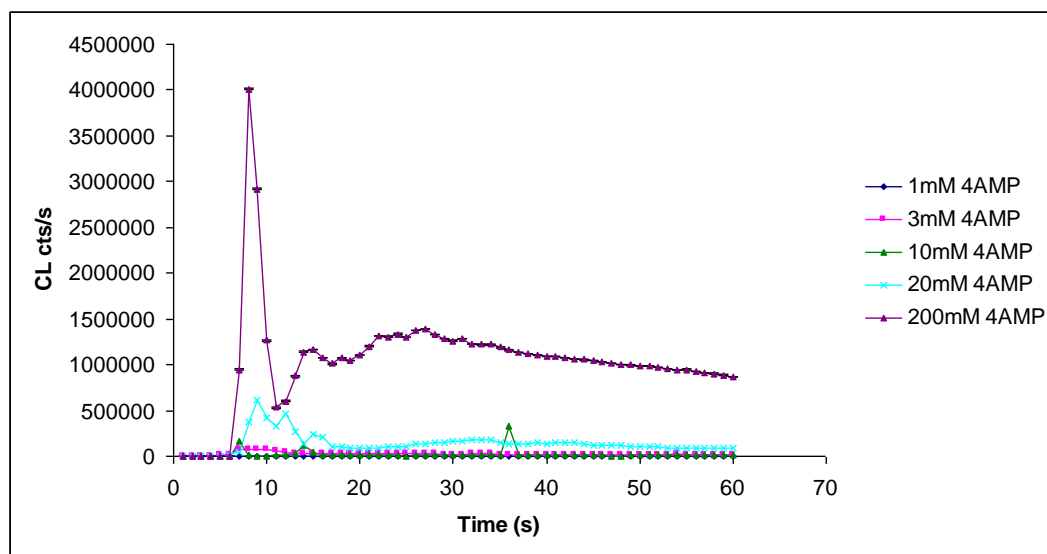
The effect of 4-aminopyridine [4-AMP] on *Obelia geniculata*. Results represent the mean of six individual hydroids ±SEM. 200mM 4-aminopyridine produced a sustained emission of light, which was maintained for over 2 minutes.1ml was injected at 3 seconds and there was a slight delay before the bioluminescent response was seen. Overall this produced more light than 1ml of 0.5M KCl.

4-AMP is probably blocking the potassium ion channels controlling the resting potential of the hydroids outer membrane. But equally the bioluminescent response may be as a result of chemical irritation or pH change. Both ion channel blockers could also be having an effect on ion channels thought to exist deeper in the proposed nerve net of the hydroid.

4.4 Discussion and future work

The main findings of this chapter were that the pattern of light emission from photocytes in *Obelia geniculata* may be different to that of *Obelia longissima.* OAS data obtained by using the Photek analysis software illustrates that in *Obelia geniculata* bioluminescent emission oscillates and can last over two minutes.

Obelia longissima shows a different pattern of light emission. There is 1 or 2 rapid peaks of photons and emission time is far shorter i.e. <30 seconds. More experiments need to be conducted to ascertain what causes this difference. A significant result was also obtained when 4-aminopyridine "a potassium ion channel blocker" was used to stimulate *Obelia geniculata*. A slightly delayed response was seen, however a very large amount of photons were emitted for a prolonged period of time. 4-aminopyridine blocks potassium ion channels in *Obelia's* membrane causing depolarisation.This rapidly results in calcium ion stimulation of the photocyte support cells probably via a nerve net. The support cells are then thought to send signals to photocytes which trigger light emission.

All three species of *Obelia* found in Pembrokeshire were shown to be bioluminescent when stimulated with potassium ions. *Obelia geniculata* from the MHPA jetty showed a higher mean bioluminescence than *Obelia dichotoma* or *Obelia longissima.* *Obelia longissima* has less photocytes per hydroid than the other two species; therefore the total light produced by each species was divided by the mean number of hydranths. This gave a more accurate comparison of the degree of bioluminescence (Fig 4.3b). This again showed that *Obelia geniculata* produced more light than the other two species. This may reflect the fact that the three species were obtained from different sample sites which may have different environmental factors. The water quality of all three sites was assessed and found to be very similar apart from a lower salinity level in Milford marina. The lower salinity in Milford marina may lower the bioluminescence of *Obelia longissima.*

The different hydroid species have different numbers and positions of photocytes and this may contribute to the differences in the amount of bioluminescence they

produce. *Obelia longissima* has one photocyte positioned just beneath each hydranth, whereas *Obelia geniculata* has many photocytes dispersed throughout the hydroids upright.

Obelia dichotoma appeared to give a broader peak of light emission upon stimulation with potassium ions, compared to the other two species, this is worthy of further investigation. Within each species the photon counts from six individual hydroids did show considerable variation in the amount of light they produced but the pattern of light production was the same. A rapid peak of bioluminescence was emitted from each live hydroid immediately K^+ was injected. This supports the hypothesis that K^+ depolarises the outer membrane of the hydroid's excitable epithelial cells. This is thought to stimulate the proposed nerve net of *Obelia* and results in calcium ions entering non-luminous support cells. Calcium ions enter the photocyte and bind to obelin, instantaneously resulting in light emission. However recording *Obelia geniculata's* bioluminescence with the Photek imaging camera and producing an avi file of bioluminescence has shown that this response can last over five minutes (avi files of these are available). This illustrates the highly sensitive detection of bioluminescence which is achieved, when using the Photek system.

The effect of lowering the concentration of potassium ions used to trigger the bioluminescent response of each hydroid was investigated. In *Obelia geniculata* 1ml of 0.5 M and 0.25 M K^+ produced a very similar degree of light emission. Decreasing the concentration of K^+ any further resulted in a much lower bioluminescent response (Fig 4.8 and 4.9). These lower concentrations of potassium ions may not be sufficient to initiate depolarisation of the hydroid's

outer membrane. O.5 M Na$^+$ produced no response above the normal background, illustrating that it is not merely the addition of any cation which produces the proposed depolarisation. In *Obelia dichotoma* and *Obelia longissima* concentrations below 0.5 M K$^+$ significantly reduced the amount of photons emitted by the hydroids. This result confirms that 0.5 M K$^+$ is the preferable concentration to use when eliciting a bioluminescent response from the hydroids. This can be utilised to stimulate specimens and record their bioluminescence either using the chemiluminometer or the Photek imaging system. The potassium ion experiments could be repeated using the Photek imaging system. Total light images measured by the Photek imaging system illustrate, that bioluminescence is emitted from the photocytes positioned in the coenosarc of *Obelia geniculata* and not from the hydranths or gonothecae. It again recorded the rapid speed of the bioluminescent response immediately that the hydroid is stimulated with potassium ions. As expected the bioluminescent response of five hydroids simultaneously stimulated produced far more photons than an individual hydroid and this light emission did not rapidly decay back to background level.

Evidence for light moving up the hydroid

To determine whether the bioluminescence is a wave of light moving up the hydroid the photon counts from ten "hot areas" were calculated. Each "hot areas" is assumed to be the position of a photocyte.

Photon emission from ten "hot areas" of *Obelia geniculata* plotted on the same axis shows scintillation of light from each photocyte. The photon emission from each photocyte is of varying intensity but the pattern of light emission is the same.

Examination of an individual "hot area" shows multiple peaks of photons lasting over 60 seconds. Calcium ions must therefore be rapidly entering and leaving the photocyte. A different pattern of light emission was found in the photocytes of *Obelia longissima*. Far less photons were emitted and the two main peaks of emission were produced within 20 seconds .Three subsequent tiny peaks are then seen. The duration of light emission is not as long as that from *Obelia geniculata.* This analysis now needs to be carried out on *Obelia dichotoma* to establish if it also has a different pattern of bioluminescent light emission.

Can the wave of light be blocked?

Soaking hydroids in 10 mM octanol was found to severely reduce their ability to produce a bioluminescent response after stimulation with potassium ions. This result agrees with experiments conducted by Dunlap et al (Dunlap et al. 1987), who hypothesised that octanol may block gap junctions thought to exist between non luminous support cells and photocytes. Alternatively the octanol may be disrupting the membrane of the hydroids perisarc.

Stimulation with the potassium ion channel blocker tetraethylammonium chloride (TEA), produced a bioluminescent response of approximately 50% of that elicited with 0.5 M K^+. 200 mM TEA gave this response but lower concentrations did not.

Stimulation with the potassium ion channel blocker 4 aminopyridine produced a bioluminescent response greater than that of 0.5M K^+ and interestingly this response did not decay back to a background level. This heightened response is worthy of further investigation. However a concentration of 200 mM was needed to produce this response. 4-AMP is probably blocking the potassium ion channels

controlling the resting potential of the hydroids outer membrane. But equally the bioluminescent response may be as a result of chemical irritation or pH change. Both ion channel blockers could also be having an effect on ion channels thought to exist deeper in the proposed nerve net of the hydroid.

Future work

The m-RNA could be extracted from the hydroids and probed for sequences which would indicate the presence of certain ion channels in the hydroids membrane. PCR could be utilised to ascertain if the message for particular channels is present. Western blot analysis would confirm if the message is being converted into real protein. Further experiments could be carried out on all three species using a range of ion channel blockers. An OAS analysis needs to be carried out on *Obelia dichotoma* to establish if it also has a different pattern of bioluminescent light emission. The effect of 4 aminopyridine on bioluminescence needs more investigation. Healthy specimens of *Obelia dichotoma* and *Obelia longissima* were not available when this experiment was initially carried out. A wider range of dilutions of 4 aminopyridine could also be used. It would also be worthwhile soaking hydroids in a blocker before trying to trigger bioluminescence with potassium ions.

Chapter five

What is the origin of coelenterazine in *Obelia geniculata?*

5.1 Introduction

Bioluminescence is utilised as a major communication system in the deep oceans. Coelenterazine is the luciferin responsible for the majority of bioluminescent reactions in the sea (Shimomura et al. 1980; Thomson et al. 1995). Coelenterazine an organic luciferin was originally discovered by Osamu Shimomura (Shimomura, 1987).

5.1.1 Diet verses *de novo* synthesis of coelenterazine

Coelenterazine is found in many other marine organisms apart from coelenterates. In fact it is the most common chemistry responsible for bioluminescence in the sea (Shimomura et al. 1980). It is known to be utilised for bioluminescence in 6 marine phyla, "Cnidarians, Ctenophores, Mollusca, Arthropoda, Chordata and Chaetognath" (Shimomura et al. 1980; Campbell & Herring, 1990; Thomson et al. 1995).

Many non-luminous organisms have been shown to contain coelenterazine. Several coelenterate bioluminescent genes have been cloned. How the marine organisms obtain the luciferin component of their bioluminescent reaction, is a matter of debate. There are two theories on the source of coelenterazine. Either the luciferin is obtained through the diet, or luminous marine organisms are capable of *de novo* synthesis of the luciferin. If the answer to this key question could be discovered it would be of major importance in understanding some of the food chains in the sea, especially those which involve cnidarians. It may well be that the solution is that both processes are occurring. From current evidence different species may obtain their luciferin in different ways and there is unlikely to be one

solution to this question. Luciferases are thought to be unique to the species they occur in and it is therefore highly unlikely that they have a dietary source. Bioluminescent organisms must have synthesising pathways to produce their luciferases (Campbell & Herring, 1990).

It has been established by several different research groups that significant levels of coelenterazine are present in a variety of non luminous marine species. These may provide a dietary source of coelenterazine for bioluminescent marine genera (Shimomura, 1987; Campbell & Herring, 1990).

5.1.2 Evidence for marine organisms obtaining imidazolopyrazine luciferins from their diet.

Haneda et al provided evidence for a dietary source of imidazolopyrazine luciferin, in some bioluminescent species. They demonstrated that *Vargula hilgendofi (Cypridina hilgendorfi)* the luminous sea fire fly was present in the stomach of *Parapriacanthus ransonneti,* a bioluminescent fish. Haneda noted that luminous ducts are connected to the digestive system of *Parapriacanthus ransonneti*. He also established that extracts from luminous organs of *Parapriacanthus* and *Apagon* cross reacted with *Vargula hilgendofi.* "The components cross reacted with extracts of *Cypridina hilgendorfi*". Haneda et al therefore concluded that *Vargula (Cypridina)* is the most likely source of luciferin for these bioluminescent fish (Haneda et al. 1966). It has been established that the midshipman fish *Porichthys notatus* is luminous in some habitats, but not in others. Similarly the hydroid *Clytia hemisphaerica* is only luminous in some parts of the world. The midshipman fish uses the *Vargula*–type luciferin which is an

153

imidazolopyrazine. Barnes et al demonstrated that when the midshipman fish is non luminous due to lack of its luciferin, bioluminescence can be regained, if it is fed or injected with *Vargula* luciferin. "Luminescence capability can be induced in non-luminous *Porichthys* by peritoneal injection or forced feeding with luciferin from *Vargula* or luminous *Porichthys*". Barnes et al used non-luminous *Porichthys notatus* from Puget Sound. Once induced with even a small amount of luciferin, luminescence was maintained for a considerable time. Interestingly injecting oxidised luciferin did not induce bioluminescence. This suggests that recycling of the products of luminescence could not be induced by this method (Barnes et al. 1973; Warner & Case, 1980). Peter Herring has shown that the distribution of the luminous ostracod *Vargula hilgendofii* corresponds with the habitats of several bioluminescent fish including *Porichthys notatus.* This supports the theory that it provides a dietary source of Vargula–type luciferin (Herring, 1982). The fact that the midshipman fish has increased luciferin levels when fed on *Vargula* is further evidence in favour of this prediction. E M Thompson et al used C^{14} labelled *Vargula*-type luciferin to establish that injected luciferin was present in the photophores of the fish after a period of time (Thompson et al. 1988). These results were supported by the work of Frank who conducted experiments on *Gnathopausia ingens* a luminous mysid, which can survive in laboratory tanks for several years. Mysids kept alive in tanks became non-luminous presumably due to lack of luciferin (they were deliberately reared on a diet of non-luminous fish). When they were fed with a source of coelenterazine, bioluminescence was restored in the majority of the mysids (Frank et al. 1984).

Shimomura has predicted that bioluminescent shrimp and fish may obtain some of their coelenterazine through diet. "In the bioluminescent shrimp and fishes, coelenterazine occurs most abundantly along the digestive tract, suggesting the possibility that this compound derives from ingested food" (Shimomura et al.1980).

A study on *Aequorea* has shown that it may lose its luminescence when appropriate food is not present (Haddock et al. 2001). Steve Haddock has experimented on *Aequorea aequorea* which have been kept in culture at Monterey Bay aquarium, California for a considerable period of time. The *Aequorea aequorea* originated from Puget Sound Washington. This "captive population" of medusae were reared in aquarium tanks and were observed to be unable to exhibit bioluminescence when mechanically stimulated. The medusae were being fed on a coelenterazine-free diet of *Artemia.* Haddock compared these with a control group of *Aequorea aequorea* netted at Friday Harbor Washington. These medusae consume plankton some of which are known to contain coelenterazine. These wild natural *Aequorea aequorea* are bioluminescent and respond to mechanical stimulation. Medusae from the wild were shown to contain photoprotein which could be extracted and produced light when stimulated with calcium ions. However this was not the case for the captive medusae. Haddock's experiments showed that the captive population did contain functional apoprotein i.e. apoaequorin. This apoprotein regenerated active photoprotein (aequorin) when incubated with coelenterazine. His results also showed that bioluminescence could be induced in the captive cohort when coelenterazine was directly injected into the medusae. Haddock concluded that *"Aequorea aequorea* is unable to produce its

155

own coelenterazine and is dependent on a dietary supply of this luciferin for bioluminescence" (Haddock, 2001).

5.1.3 The evidence for marine organisms synthesising coelenterazine.

Many species of decapods are bioluminescent and one suggested source of their luciferin is from their diet. "Oceanic decapods take a wide variety of food, much of it bioluminescent species" (Roe, 1984; Thomson et al. 1995b). Some decapods exhibit both photophore and secretory bioluminescence (refer to applications of bioluminescence in chapter 1). Coelenterazine was first detected in decapods by Inoue et al (Inoue et al. 1976). This work was followed by two groups of researchers identifying coelenterazine as the luciferin in bioluminescent Oplophoridae and Sergestidae decapods (Shimomura et al. 1980; Campbell & Herring, 1990).

Photophore and secretory bioluminescence requires relatively large quantities of luciferin, and it is highly unlikely that decapods could obtain all their luciferin from dietary sources. Thomson et al have established that coelenterazine levels increase during the life cycle of 3 species of Oplophoridae (secretory decapods). This relatively large increase in coelenterazine during the decapod life cycle supports the hypothesis of Thomson, Herring and Campbell that "the Oplophoridae must be capable of *de novo* synthesis of coelenterazine" (Thomson et al. 1995).

Thomson et al examined a range of adult decapod species to try to establish if adult decapods could synthesise coelenterazine. They were also trying to establish

the exact cellular location for biosynthesis of coelenterazine in decapods. They demonstrated that in the Oplophoridae coelenterazine is the luciferin in both photophore and secretory bioluminescence.

Bioluminescent Oplophoridae were found to contain far more coelenterazine than other families of decapods (mean value = 154 pmol per decapod). However even higher concentrations of coelenterazine were found in 11 decapod species considered to be non-luminous (mean value = 200 pmol per decapod). Coelenterazine had also previously been identified in non-luminous decapods by two other studies (Shimomura et al. 1980; Campbell & Herring, 1990).

Thomson et al measured coelenterazine levels and luciferase activity in a range of decapods. When measuring coelenterazine in decapod stomachs they emptied the stomach prior to the assay to remove dietary sources of luciferin. Significant levels of coelenterazine were found in the stomach and hepatopancreas of a limited number of species of decapod. This suggests that the stomach and hepatopancreas are sites for synthesis or storage of coelenterazine. The genes controlling this proposed synthetic route have yet to be discovered. The biosynthetic pathway needs to be clearly identified and characterised (Thomson et al. 1995).

Thomson et al also established that the eggs of the bioluminescent mid water shrimp *Systellaspis debilis* are capable of *de novo* synthesis of coelenterazine. This was the first demonstration of biosynthesis of coelenterazine in any marine species. Eggs of *Systellaspis debilis* were assayed for coelenterazine content at different stages of their development. The use of the eggs allowed coelenterazine levels to be determined without any dietary source contributing.

157

"An increase of almost two orders of magnitude was detected in coelenterazine content per egg between the first and final stage of development (mean value1 pmol and 71 pmol)". The assay used recombinant apoaequorin (Thomson et al. 1995).

More recently in 2009 Oba and Kato have used deuterium labelled amino acids and electrospray ionisation-ion trap-mass spectrometry to show that the luminous copepod *Metridia pacifica* is able to synthesise coelenterazine. They have demonstrated that the copepod will synthesise coelenterazine from one molecule of L-phenylalanine and two molecules of L-tyrosine (Oba & Kato et al. 2009). These results support the hypothesis that coelenterazine is synthesised by specific marine organisms via a cyclisation of three amino acids FYY (phenylalanine, tyrosine, tyrosine). It has been proposed that the tripeptide undergoes dehydrogenation and decarboxylation before cyclising (refer to Fig 1.6). This proposed mechanism is similar to the cyclisation of the chromophore in green fluorescent protein (GFP) from SYG (serine, tyrosine, and glycine) and the yellow and red chromophores now unravelled in corals (Campbell, 1988; McCapra, 1990). Vargula luciferin is already known to be synthesised from 3 amino acids arginine, isoleucine and tryptophan (Campbell, 1988; Kato et al. 2007). William Ward noted in 1994 that coelenterazine synthesis from phenylalanine, tyrosine, and tyrosine is almost identical to how GFP is generated from the tri peptide serine, tyrosine, and glycine. He then developed a hypothesis to suggest that the GFP gene must have evolved from a coelenterazine gene, because only one single DNA base substitution was required to change FYY to FSY (Prasher et al. 1992; Ward, 2006; Ward et al. 1998).

The first aim of this chapter was to firstly investigate whether *Obelia* requires a dietary source of coelenterazine to maintain its bioluminescence. This would test the prediction of Dr Steven Haddock that Cnidarians are unlikely to be able to synthesise coelenterazine. The second aim was to identify possible dietary sources of coelenterazine in zooplankton at *Obelia* sample sites, and to discover whether marine organisms living near, on or in *Obelia* colonies contain coelenterazine. This would test the hypothesis of Campbell and Herring that copepods could act as a potential dietary source of coelenterazine for many bioluminescent organisms including hydroids (Campbell & Herring, 1990).

The strategy involved setting up cultures of Obelia in seawater tanks and monitoring their bioluminescence over several weeks. Several zooplankton trawls were carried out at the same locations as some Pembrokeshire *Obelia* colonies. Copepod species in these trawls were identified and some tested for coelenterazine content. The coelenterazine content of the 3 species of *Obelia* was measured using the coelenterazine assay. Species living on and in the *Obelia* colonies were identified, and some tested for their coelenterazine content. Apoobelin levels were also measured in some *Obelia* colonies to ascertain if a lack of apoobelin could limit the degree of bioluminescence.

5.2 Methods

5.2.1 Culturing *Obelia geniculata*

A culture system for *Obelia geniculata* was set up. *Obelia geniculata* hydroids were dissected from *Laminaria* and initially held in place on plastic plates with cotton thread. The plastic plates were immersed in a tank of filtered seawater. In

some cultures new stolons appeared but did not stick down and therefore the culture failed. In other cultures after approximately 7 days new stolons appeared, and did in time adhere to the plastic plate. However of 8 plates set up, less than half produced new stolons, but this was enough to start new colonies of *Obelia geniculata* growing in the tank. A very slow air pump was used to circulate seawater over the hydroids, otherwise excrement collects around the hydranths and the culture died. If the air pump flow was too high it prevented new stolons forming. The culturing was based on a method by Sears Crowell which was used to culture *Laomedea flexuosa* (Crowell, 1953) and original methods used at Plymouth MBA for growing hydroids (Rees & Russell, 1937). *Obelia* is notoriously difficult to culture compared to other hydroids and some cultures were successful, whereas others failed. Cultures were maintained at $10\ ^{0}C$. The tank was rocked on a rocker to simulate wave action. Small pebbles from the beach were used to weight down the plastic plate holder; this prevented it moving around the tank. *Artemia* were also cultured to feed the hydroids. Filtered seawater was replaced on a daily basis. Plates were removed from the tank and placed in a small tank of seawater containing *Artemia* for 2hrs so that the hydroids could feed. *Artemia* had to be one to two days old. Once the *Artemia* are over 2 days old they will eat the hydroid rather than becoming its prey.

Fig.5.1. *Obelia geniculata* **in the culture tank.**

5.2.2 Zooplankton sampling

To identify and analyse possible sources of coelenterazine in the food chain of *Obelia geniculata* and other luminous hydroids, zooplankton trawls have been carried out at some sample sites. A zooplankton sampling net with a mesh of 0.3mm was deployed from the boat and the identity of the zooplankton established using microscopy. Images of some of the zooplankton including medusa have been recorded. Some batches of copepods from plankton trawls have been analysed for coelenterazine using the coelenterazine assay. Amphipods, nudibranchia and polychaeta which cohabit on and within *Obelia* colonies can also be obtained by removing them from a fresh hydroid sample under the binocular microscope using fine forceps. Using this method a polychaete *Myrina prolifer* was found living on and in the *Obelia geniculata* colonies.

Fig.5.2. Zooplankton sampling.

5.2.3 Photographing zooplankton

Most images of zooplankton were obtained at the plankton identification course at Dale Fort field studies centre. These were taken using a Nikon Coolpix 400 attached to a binocular microscope or a Moticam 1SP1.3 digital camera supplied by Timstar, Timstar house, Marshfield bank, Crewe, Cheshire, CW2 8UY. A Lumix DMC-L10K Panasonic digital camera was also used for some images. Participants in the workshop shared all the images from zooplankton trawls. I am grateful to Alan Bowden for technical expertise in capturing the image of *Tomopteris* and *Hipopolyte varians*.

5.3 Results

5.3.1 Studies on cultures of *Obelia geniculata*.

Bioluminescence over time was measured in cultures of *Obelia geniculata*. In some cultures the levels of the photoprotein obelin over time were also monitored. The effect of adding coelenterazine to a culture of *Obelia geniculata* was also investigated.
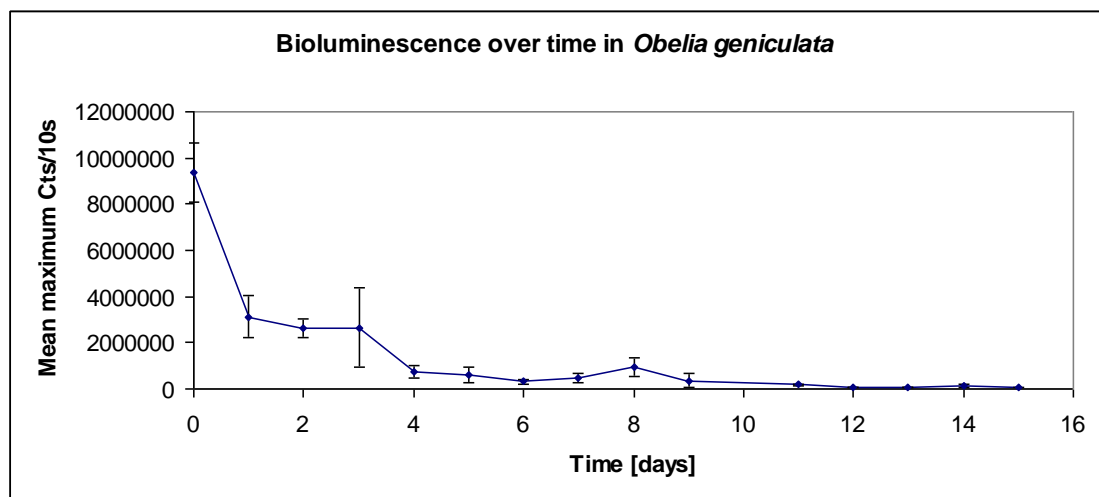
Fig.5.4. Bioluminescence over time in *Obelia geniculata*. Results represent the mean bioluminescence ± SEM of 6 hydroids measured over time in a culture of *Obelia geniculata* in July.

Bioluminescence faded, and by day 15 the hydroids had lost all their bioluminescence. The hydroids were kept alive by daily feeding with *Artemia (*brine shrimp*)*.
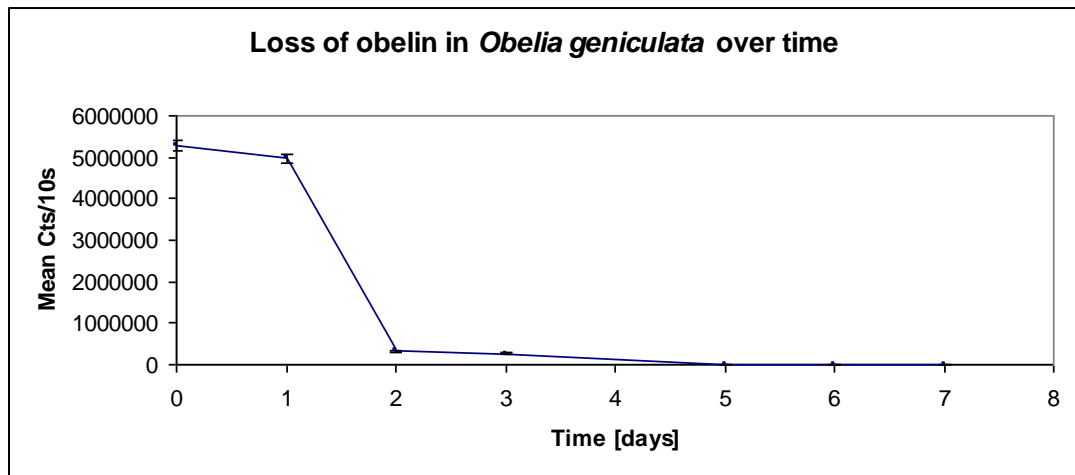
Fig.5.5. Loss of obelin activity in a culture of *Obelia geniculata* in August.
Results represent the mean of 6 measurements of photoprotein activity ± SEM.
Obelin activity fell dramatically after two days. This is most likely due to lack of a
dietary source of coelenterazine.
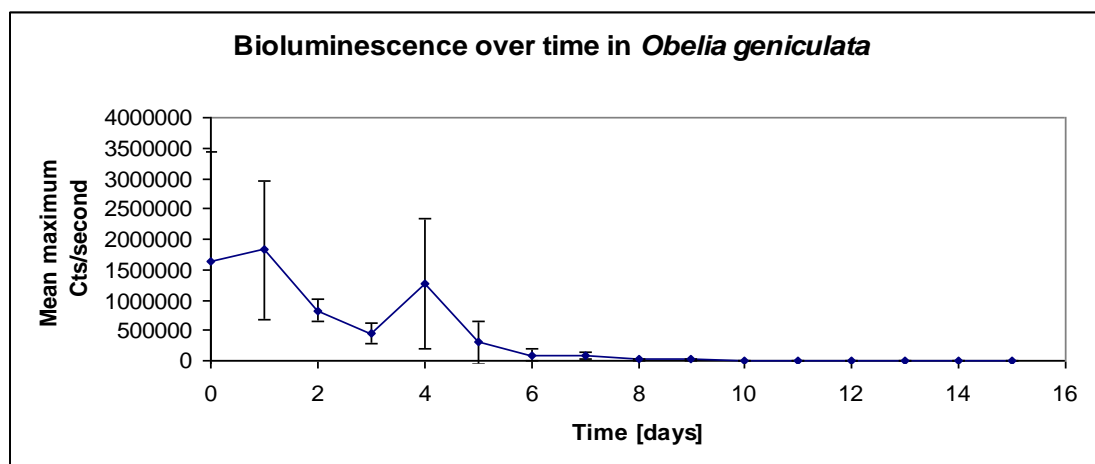
Fig.5.6. Mean Bioluminescence of 6 hydroids ± SEM measured over time in a
second culture of *Obelia geniculata* in August. In this culture bioluminescence
rapidly deteriorated .This corresponded with a dramatic drop in photoprotein
activity in the same culture (Fig 5.5). Bioluminescence briefly recovered on day 4.

Some cultures of *Obelia geniculata* remained bioluminescent for 9 days whilst in

others mean bioluminescence plummeted after only 3 to 4 days. In the August

culture (Figs 5.4 and 5.5) a loss of bioluminescence was accompanied by a rapid

loss of photoprotein activity. The most likely cause of this loss of bioluminescence

is lack of a dietary source of coelenterazine or some other vital factor required by

Obelia geniculata to manufacture its own coelenterazine. The bioluminescence of

both culture's bioluminescence did fluctuate (Fig 5.5 and Fig 5.6), indicating that

Obelia geniculata might be capable of *de novo* synthesis of coelenterazine, or

recycling oxidised luciferin. These results are worthy of further investigation.
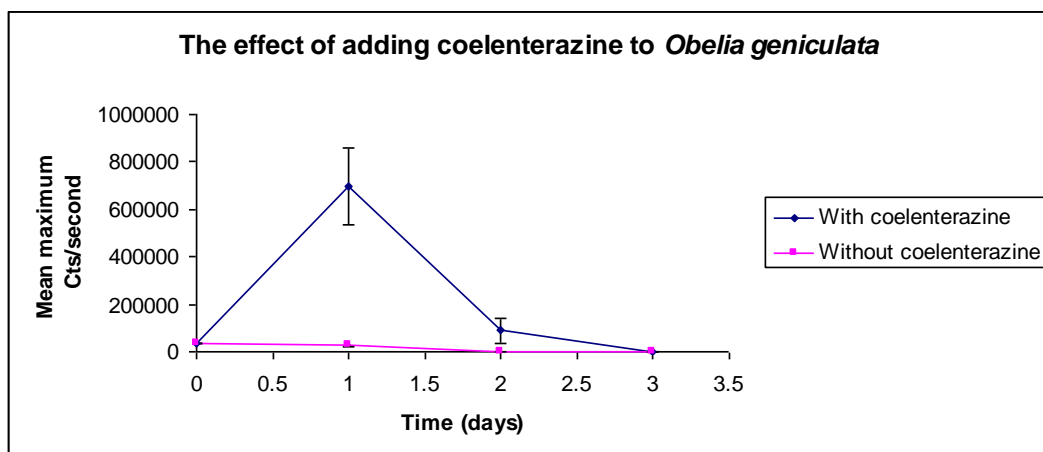
Fig.5.7 The effect of adding coelenterazine. This was measured by adding coelenterazine to a culture of *Obelia geniculata* that had previously lost nearly all its bioluminescence. Results were the mean maximum counts sec^{-1} from 4 individual hydroids ± SEM. Hydroids that had no coelenterazine added to them were used as a control.

Adding coelenterazine to a culture of *Obelia geniculata*, which had previously lost

its bioluminescence did restore a low level of luminescence. This effect was very

short lived. Further experiments need to be conducted to establish if

bioluminescence can be restored to a colony using this or a similar method.

5.3.2 Species identified in zooplankton trawls

Zooplankton trawls were carried out at the same location, as some of the *Obelia geniculata* colonies in Pembrokeshire waters.

Class	Species	Luminous	% in trawl
Hydrozoan (Medusa)	*Obelia lucifera* *	yes	7.83
Hydrozoan (Medusa)	*Phialidium hemisphaerica* *	yes	2.61
Hydrozoan (Medusa)	*Phialella quadrata*	no	6.10
Copepoda	*Centrophages typicus* **	no	6.96
Copepoda	*Oithona helgolandia* **	no	4.35
Copepoda	*Acartia clausi* **	no	10.43
Decapods	*Anapagarus hyndmanni*	no	3.48
Decapods	*Pisidia longicormis*	no	1.74
Decapod larva	*Carcinus maenus*	no	13.04
Polychaeta	*Tomopteris helgolandia* *	yes	7.83
Polychaeta	*Myrianida prolifer* **	no	9.57
Cirripedia	*Balanus crenatus*	no	11.30
Ctenophora	*Pleurobrachia pileu*s	yes	13.04
Ctenophora	*Beroe cucumis*	yes	1.74

Table.5.1 Species identified from a zooplankton trawl at Watwick point, where colonies of *Obelia geniculata* grow on *Laminaria*. Species marked * have previously been recorded as containing coelenterazine. Species marked ** were found in this research, to contain low levels of coelenterazine (refer to result tables 5.7 and 5.8).

Fig.5. 8. *Phialella quadrata* a non-luminous Cnidarian medusa. Black and white image recorded with moticam and binocular microscope on high power.
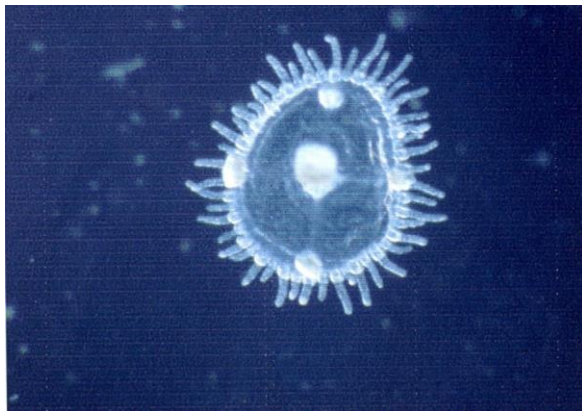
Fig.5. 9 *Obelia lucifera* the luminous medusa from *Obelia geniculata* identified from a zooplankton trawl at Watwick point. Black and white image recorded with Nikon cool-pix, and binocular microscope on high power.

Fig.5.10. The non-luminous copepod *Acartia clausi* which was present in some of the zooplankton trawls. This species is very common in Pembrokeshire estuaries and seas. Image recorded with moticam and binocular microscope on high power

No bioluminescent species of copepods were found. The 3 species of copepoda identified were tested for coelenterazine content using the coelenterazine assay. The medusa of *Obelia geniculata* was in the plankton together with the polychaeta *Myrianida prolifer* known to live in and on *Obelia* colonies. Four bioluminescent species in total were in the trawl from Watwick point.

Fig.5.11. *Myrianida prolifer* a non-luminous polychaeta which was present in all zooplankton trawls. Image recorded with Panasonic camera and binocular microscope on high power.

Fig.5.12. Decapod *Hipopolyte varians* from zooplankton trawl at Watwick point. Image recorded using the binocular microscope on high power and Nikon coolpix camera.

Copepoda found on larger marine organisms at Watwick point.

Copepod species	Source
Caligus minimus	in throat of sea bass
Lernanthropus kroyeri	in gills of sea bass
Caneerila tubulata	on *Amphipholas squamata*

Table. 5.2 Three additional species of copepods were found, when dissecting a sea bass caught at Watwick point and a brittle star *Amphipholas squamata* blocking the zooplankton net. All three species are thought to be parasitic and non-luminous.

Species in zooplankton trawl at Burton Ferry

Class	Species	Luminous	% in trawl
Hydrozoan (Medusa)	*Bougainvillia* sp	no	2.29
Hydrozoan (Medusa)	*Phialella quadrata*	no	3.82
Copepoda	*Calanus* sp	no	1.53
Copepoda	*Temora longicornis*	no	5.34
Cirripedia	*Balanus crenatus*	no	9.92
Cirripedia	*Semibalanus balansides*	no	5.34
Decapods larva	*Carcinus maenus*	no	6.87
Decapods	*Pisidia longicormis*	no	3.82
Decapods	*Hipopolyte varians*	no	1.53
Amphipoda	*Chaetogammurus marinus* **	no	11.45
Amphipoda	*Phtisica marina*	no	8.40
Amphipoda	*Caprella equilibra*	no	4.58
Polychaeta	*Tomopteris septentrionalis* *	yes	6.11
Polychaeta	*Myrianida prolifer* **	no	10.69
Larvacean	*Oikopleura dioica*	no	2.29
Malacostraca	*Pseudocuma longicornis*	no	2.29
Ctenophora	*Pleurobrachia pileus*	yes	12.98
Ctenophora	*Beroe cucumis*	yes	0.76

Table.5.3. Species identified from a zooplankton trawl at Burton ferry where colonies of *Obelia geniculata* grow on *Laminaria*. Species marked * have previously been recorded as containing coelenterazine. Species marked ** were found to contain low levels of coelenterazine (refer to result tables 5.7 and 5.8).

Fig.5.13. *Chaetogammurus marinus*. This non-luminous amphipod was present in zooplankton trawls at Martin's Haven, Burton Ferry and Milford Haven marina. It was also found living on *Obelia* colonies at Milford Haven port authority jetty. Image captured with moticam and binocular microscope on high power.

Fig 5.14 *Tomopteris* a luminous polychaete found in trawls at all three zooplankton sample sites. Image captured with using the binocular microscope on high power and Nikon coolpix camera.

Harvey reported that this polychaete produced bioluminescence "using a luciferin-luciferase reaction" (Harvey 1952; Shimomura, 2006). Shimomura's limited research on *Tomopteris* suggests it may possess a photoprotein system resembling that of *Pholas dactylus* and *Chaetopterus* (Shimomura, 2006).

Species in zooplankton trawl at Martin's Haven

Class	Species	luminous	% in trawl
Cnidarian medusa	*Obelia lucifera* *	yes	4.61
Copepoda	*Centrophages typicus* **	no	3.29
Copepoda	*Acartia clausi* **	no	8.55
Copepoda	*Temora longicornis*	no	1.97
Amphipoda	*Chaetogammurus marinus* **	no	15.13
Amphipoda	*Phtisica marina*	no	12.50
Decapod larva	*Carcinus maenus*	no	11.84
Polychaeta	*Tomopteris helgolandia* *	yes	4.61
Polychaeta	*Myrianida prolifer* **	no	5.92
Cirripedia	*Balanus crenatus*	no	17.11
Cirripedia	*Balanus crenatus* (euvia)	no	3.29
Ctenophora	*Pleurobrachia pileu*s	yes	9.21
Ctenophora	*Beroe cucumis*	yes	1.97

Table.5.4. Species identified in zooplankton trawl at Martin's Haven. Species marked * have previously been recorded as containing coelenterazine. Species marked ** were found to contain low levels of coelenterazine (refer to result tables 5.7 and 5.8).

Class	Species	Luminous	% in trawl
Amphipoda	*Chaetogammurus marinus*	no	38.03
Amphipoda	*Phtisica marina*	no	12.68
Decapod (larva)	*Carcinus maenus*	no	15.49
Polychaeta	*Myrianida prolifer* (with eggs)	no	11.27
Cirripedia	*Balanus crenatus*	no	18.31
Cirripedia	*Balanus crenatus* (euvia)	no	4.23

Table.5.5. Zooplankton trawl from Milford Haven marina, where colonies of *Obelia longissima* exist. Far fewer species were present in this trawl. Species marked ** were found to contain low levels of coelenterazine (refer to result tables 5.7 and 5.8).

Fig 5.15 *Carcinus maenus* a non-luminous decapod larva commonly called megalopa. Image captured with using the binocular microscope on high power and Nikon coolpix camera.

Fig5.16. A luminous amphipod *Phtisica marina* found in zooplankton trawls, and also living on *Obelia* colonies. Image captured with Panasonic camera and binocular microscope under high power.
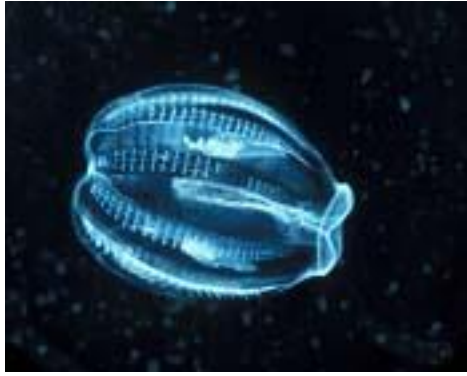
Fig.5.17. *Pleurobrachia pileu*s a luminous ctenophora commonly called the sea gooseberry.

Bioluminescent marine crustaceans include; ostracods, copepods, amphipods, euphausiids and decapods. These can all be found in zooplankton samples and some are possible candidates to be dietary sources of coelenterazine for hydroids. Considering the size of zooplankton prey, which hydroids can consume, their most likely dietary source of coelenterazine are copepods, ostracods, amphipods or euphausiids. Nearly all known euphausiids are thought to be luminous e.g. *Euphausia pacifica* and *Meganyctiphanes norvegica.* They are commonly referred to as krill. They have been found to utilise a tetrapyrole luciferin and are not known to contain coelenterazine. There are two major orders of copepods Cyclopoida and Calanoida. Ten bioluminescent species of copepod are known (Herring, 1988; Harvey, 1952), including; *Cyclopoida Oncaea, Calanoid Metridia , Calanoida Pleuromamma, Calanoida Gaussi.* Five copepod genera are known to utilise coelenterazine as the luciferin in their bioluminescence (refer to Table 5.6).

Class	Genus
Copepoda	*Euaugaptilus*
	Disseta
	Pleuromamma
	Hemirhabdus
	Lucicutia
Ostracoda	*Conchoecia*
	Vargula
Decapoda	*Acanthephyra*
	Oplophorus
	Systellaspis
	Heterocarpus
	Sergestes
	Hymenodora
Mysidacea	*Gnathophausia*

Table.5.6. Marine genera in zooplankton known to use imidazolopyrazine based bioluminescence. All are thought to use coelenterazine as a luciferin, apart from *Vargula* which uses *Vargula*-type luciferin. Adapted from Campbell & Herring, (1990).

No bioluminescent copepods were identified in the four areas that zooplankton trawls were carried out. Older records of marine organisms in the Dale Fort fauna record 89 different species of copepods in Pembrokeshire waters, but only one of these may refer to a bioluminescent species. *Metridia lucens* was recorded in deeper water at night in the spring of 1960 and this may refer to the bioluminescent copepod species. No ostracods were detected in the recent

zooplankton trawls. Only two species of ostracod are recorded in the Dale Fort marine fauna as historically being present in the sea around Watwick point. One of these is *Vargula* but this would be a source of *Vargula*-type luciferin rather than coelenterazine. The coelenterazine containing ostracod *Conchoecia* was not recorded. Several species of decapods were present in the trawls. Decapods include decapod shrimp, which are identified by the fact that they "have 3 thoracic appendages which they use as limbs as well as five leg like appendages". Many decapod shrimp are luminous e.g. *Systellaspis* and their larvae are another possible source of coelenterazine for hydroids (Thomson et al. 1995). Unfortunately no specimens of *Systellaspis debilis* a decapod known to contain coelenterazine were found. This species is not recorded in the Dale Fort fauna. It is known to favour deeper ocean waters.

5.3.3 Coelenterazine content of copepods

Copepod species	Coelenterazine content mols
Centrophages typicus	2.91×10^{-15}
Oithona helgolandia	1.00×10^{-15}
Acartia clausi	1.40×10^{-15}
Calanus sp	not detected
Temora longicornis	not detected

Table.5.7. Copepods in the zooplankton trawls were tested for coelenterazine content using the coelenterazine assay. Results represent the mean of 3 measurements of coelenterazine concentration per organism ± SEM.

No Coelenterazine was detected in two of these non bioluminescent species. Relatively low levels of coelenterazine were detected in the three other copepod species.

This result is not surprising as none of the copepod species identified in the trawls was a bioluminescent one, or one that had previously been found to contain coelenterazine. *Calanoid Metridia* is a bioluminescent copepod species sometimes found in British waters and known to contain coelenterazine, but it was not present in any of the trawls. There is one record in the Dale Fort fauna which may refer to it as *Metridia lucens.* Dr David Copeland at Plymouth MBA has recorded *Calanoid Metridia* in a few zooplankton samples taken at a Plymouth off shore sampling area. It might be possible to obtain samples of *Calanoid Metridia*, in Pembrokeshire waters if trawls are conducted further out to sea.

Coelenterazine content of *Obelia* hydroids, *Myrina prolifer* (a polychaeta) and *Chaetogammurus marinus* (an amphipoda)

Species	Coelenterazine content mols
Myrina prolifer	3.05×10^{-15}
Chaetogammurus marinus	3.34×10^{-15}
Obelia dichotoma	0.96×10^{-13}
Obelia geniculata	3.36×10^{-12}
Obelia longissima	5.95×10^{-15}

Table.5.8. Coelenterazine content detected in *Obelia* species and two other species which exist on and in the *Obelia* colonies. Results were obtained using the coelenterazine assay. The polychaete living inside *Obelia geniculata, Myrina prolifer* was found to contain a low level of coelenterazine together with *Chaetogammurus marinus* which lives on the colony. The results represent the mean of three observations ± SEM.

Chaetogammurus marinus and *Myrina prolifer* were observed in and on *Obelia* colonies. *Chaetogammurus marinus* was found to live inside cocoons on *Obelia longissima. Chaetogammurus marinus* and *Myrina prolifer* were also observed consuming the hydranths of *Obelia.* They are probably obtaining coelenterazine by eating *Obelia.* More experiments need to be carried out to confirm this. Both these species have not been previously recorded as containing coelenterazine. The levels discovered in these two species are relatively low. Juvenile amphipods and polychaetes may contain coelenterazine and could provide a dietary source of coelenterazine, if they are consumed by *Obelia*. Further experiments are needed to

test this hypothesis. *Obelia geniculata* and *Obelia dichotoma* had higher coelenterazine content than *Obelia longissima.*

The level of coelenterazine in *Obelia geniculata* is consistent with those found by Thomson in the same hydroid which ranged from 0.07 to 12.03 pmol per hydroid (Thomson, 1995). Campbell and Herring however found a lower level of 0.33 pmol per hydroid, in *Obelia geniculata* (Campbell & Herring, 1990). *Obelia dichotoma* and *Obelia longissima* have not been previously tested for coelenterazine. The far lower level of coelenterazine in *Obelia longissima* could be due to lack of coelenterazine containing prey in the marina habitat. The lower level of coelenterazine in *Obelia dichotoma* compared to *Obelia geniculata* could be due to environmental differences.

Ratio of obelin to apoobelin in *Obelia* colonies

Colony species and location	Ratio of obelin to apoobelin In sample taken in June	Ratio of obelin to apoobelin In sample taken in September
Obelia geniculata colony at Milford Haven port authority jetty.	3:1	1 : 1.5

Table.5.9. Ratio of obelin to apoobelin in a colony of *Obelia geniculata* colony at Milford Haven port authority jetty.

Colony species and location	Ratio of obelin to apoobelin In sample taken in May	Ratio of obelin to apoobelin In sample taken in September
Obelia longissima colony at Milford Haven marina	4:1	1:7.5

Table.5.10. Ratio of obelin to apoobelin in a colony of *Obelia longissima* colony at Milford Haven marina.

In both *Obelia* species tested, ratios of obelin to apoobelin were found to alter in older colonies at the end of the hydroid season, when compared to young colonies at the start of the season. In new colonies there is x 3 to 4 more obelin than apoobelin. In older colonies of *Obelia geniculata* there are close to equal levels of obelin and apoobelin. In older colonies of *Obelia longissima* there was far more apoobelin than obelin. This colony was very old and therefore loss of photoprotein may be part of the ageing process. An alternative theory is that lack of coelenterazine containing plankton in September influences the production of photoprotein.

5.4 Discussion and future work

The main findings of this chapter are that cultures of *Obelia geniculata* fed on non luminous *Artemia* rapidly lost their bioluminescence, indicating that the hydroid requires a dietary supply of coelenterazine. The mean bioluminescence of *Obelia geniculata* cultures was found to fluctuate over a period of days, which suggests *Obelia* might be able to manufacture coelenterazine. Adding coelenterazine to a non bioluminescent culture of *Obelia* was found to briefly restore its bioluminescence. A lower level of coelenterazine was found in *Obelia longissima*, when compared to *Obelia geniculata* and *Obelia dichotoma.* Low levels of coelenterazine were also detected in *Myrina prolifer* which grows inside the *Obelia* colony and in *Chaetogammurus marinus* an amphipod commonly found living on the *Obelia* colonies. Three non luminous species of copepod present in the zooplankton were found to contain low levels of coelenterazine. No coelenterazine containing bioluminescent copepods were found in a number of

zooplankton trawls conducted in waters above *Obelia* colonies. In *Obelia geniculata* and *Obelia longissima* colonies ratios of obelin to apoobelin were found to alter in older colonies at the end of the hydroid season, when compared to young colonies at the start of the season. As the colonies aged, the levels of apoobelin increased.

Cultures of *Obelia geniculata* fed on non luminous *Artemia* rapidly lost their bioluminescence. These results achieved the primary aim of this chapter to investigate whether *Obelia* requires a dietary source of coelenterazine, to maintain its bioluminescence. These results are worthy of further investigation. The mean bioluminescence of *Obelia geniculata* cultures was found to fluctuate over a period of days, which may be evidence of *Obelia* being able to manufacture coelenterazine. Levels of the photoprotein obelin also dramatically fell in these cultures. Adding coelenterazine to a non bioluminescent culture did briefly restore some bioluminescence. These results support the hypothesis of Dr Steven Haddock that all Cnidarians require a dietary source of coelenterazine to produce their bioluminescent response. Haddock studied non luminous *Aequorea aequorea* raised in an aquarium. He contrasted these medusae with the natural bioluminescent population of *Aequorea aequorea* in Friday Harbor Washington. Haddock found no active photoprotein (i.e. aequorin) in *Aequorea aequorea* which he reared in an aquarium. He did extract active apoprotein from these "captive medusae" confirming that it was lack of coelenterazine which prevented the medusae from being bioluminescent. Haddock established that injecting coelenterazine in methanol directly into *Aequorea aequorea* immediately restored its bioluminescence. Further experiments on *Obelia geniculata* cultures need to be

conducted to monitor, whether direct injection of coelenterazine into the hydroids will restore their bioluminescence. Levels of bioluminescence, photoprotein, and apoprotein could be monitored in these hydroids. The question as to whether *Obelia geniculata* is capable of coelenterazine biosynthesis remains unanswered. Haddock predicts that Cnidarians as a whole are unlikely to be able to synthesise this luciferin "Perhaps no Cnidarian is able to produce it and the chemical may actually be misnamed" (Haddock, 2001).To date only two species have been shown to be capable of synthesising coelenterazine. These are *Systellaspis debilis* (a decapod) and *Metridia pacifica* (a copepod), neither are Cnidarians (Thomson et al. 1995; Oba & Kato et al. 2009). Haddock's work was preceded by the experiments of other notable scientists, who established that other marine species require a dietary source of coelenterazine including *Porichthys notatus* (Herring, 1982). The evidence for a dietary source of coelenterazine has previously been discussed in detail in 5.1.2. Different marine species may obtain their coelenterazine by a combination of diet and synthesis. There is unlikely to be one overall answer to the source of coelenterazine. Some previous experiments have discovered that relatively tiny amounts of luciferin produced a long lived bioluminescent capability in some species (Barnes et al. 1973). The presence of luciferin in the stomach may be "switching on" biosynthetic pathways. Shimomura has predicted that some marine organisms may convert a store of sulphated coelenterazine into coelenterazine, as opposed to synthesising the luciferin; however there is no experimental evidence to suggest that Obelia contains sulphated coelenterazine (Shimomura, 2006).

Copepods are one of the most abundant species in zooplankton (Herring, 1988). Herring and Campbell have proposed that they are a very likely source of coelenterazine for many luminous marine species. They have previously used the coelenterazine assay to detect coelenterazine levels ranging from 2 to 35 pmol per copepod in seven different bioluminescent copepod species. They also detected coelenterazine in the non-luminous copepod *Pareuchaete as* represented in Table 5.11 (Campbell & Herring, 1990).

Copepod species found to contain coelenterazine
Euaugaptilus magns
Euaugaptilus laticeps
Euaugaptilus peodiosus
Pleuromamma xiphias
Lucicutia spp
Megacalanus princeps
Hemirhabdus grimaldi
Pareuchaete spp

Table 5.11 Copepod species found by Campbell and Herring to contain coelenterazine. All are bioluminescent species other than *Pareuchaete* (adapted from Campbell & Herring, 1990).

Unfortunately none of these coelenterazine containing species was present in the zooplankton trawls carried out in Pembrokeshire waters. The historical records of copepods found in Pembrokeshire waters do not record any of these species (Crothers, 1966). Campbell and Herring obtained their copepods from "mid water trawls" using RRS Discovery, during a cruise in the North Atlantic Sea (cruise

120). Dr Catherine Thomson found coelenterazine in 12 copepod species. Levels ranged from 0.15 to 277 pmoles. A very high level of coelenterazine was found in the species *Gaussia princeps* i.e. 277 pmoles per copepod. She also detected coelenterazine in 5 species of non-luminous copepods table 5.12 (Thomson, 1995). Thomson used RRS Discovery to obtain copepods from "mid water trawls" and performed on board coelenterazine analysis. The trawls were performed in the seas of the West African coast and Arabian Sea. None of these copepod species found by Thomson to contain coelenterazine were present in the zooplankton trawls carried out in Pembrokeshire waters.

Copepod species found to contain coelenterazine by Thomson
Euaugaptilus magns
Lucicutia sp
Hemirhabdus sp
Pleuromamma sp
Gaussia princeps
Megacalanus sp
Bathycalanus*
Pareuchaete sarsii*
Euchirella sp*
Euchaeta sarsii*
Oncaea conifera
Valdiviella*

Table 5.12 Twelve Copepod species found to contain coelenterazine by C. Thomson. Those marked * are non luminous (adapted from Thomson, 1995).

The historical records of copepods found in Pembrokeshire waters do also not record any of these species (Crothers, 1966). Low levels of coelenterazine were found to be present in three non bioluminescent copepod species during this research .The levels were not as high as those detected by Thomson in different species of deep water non luminous copepods. These results are worthy of further investigation. Shimomura has previously found low levels of coelenterazine in other non luminous marine species (Shimomura, 1987; Shimomura, 2006). Therefore coelenterazine containing species of copepods remain a possible dietary

source of luciferin for bioluminescent hydroids, but more zooplankton trawls, at varying depths need to be carried out to ascertain if coelenterazine containing bioluminescent copepods are in Pembrokeshire waters. Thomson's previous results showing possible levels of up to 277 pmol per copepod, illustrate that copepods could provide a sufficiently high concentration of coelenterazine for *Obelia* to utilise. Further investigations need to be carried out to ascertain whether these species do live in the relatively shallower coastal waters, where some Pembrokeshire *Obelia* colonies are known to exist, or whether they are confined to deeper waters. Some evening trawls could be conducted, as some species come nearer the surface at night. Two species of copepods found in zooplankton trawls in Pembrokeshire did not contain significant levels of coelenterazine. This was not unexpected, as none of the five species found has previously been recorded as containing coelenterazine. They were not the same species as those investigated by Thomson or Herring and Campbell (Thomson, 1995; Herring & Campbell, 1990). If *Calanoid Metridia* is only found off shore it is unlikely to be a source of coelenterazine for *Obelia* colonies closer to the shore.

More zooplankton trawls need to be carried out at a variety of depths to finally ascertain if any of the bioluminescent coelenterazine containing copepod species do exist close to the known sites of *Obelia* colonies in Pembrokeshire. Shimomura discovered that non luminous marine species can contain coelenterazine as well as bioluminescent species. Despite the fact that these non-luminous species contain relatively low levels of coelenterazine, he predicted that such species could provide "a supplementary dietary supply of coelenterazine" (Shimomura, 1980). If coelenterazine containing copepods are found in further trawls, daily consumption

186

of these by hydroids may be providing a coelenterazine source, as predicted by the hypothesis of Campbell and Herring (Campbell & Herring, 1990). There are unlikely to be many deep water copepods in the waters of Milford Haven marina, where colonies of *Obelia longissima* exist. The results of the zooplankton trawls do meet the original aim of identifying possible dietary sources of coelenterazine that Obelia colonies could utilise. More of the species found in the trawls need to be assayed for coelenterazine content. Further trawls in the marina may reveal juvenile amphipods, decapods or even polychaeta present in the marina zooplankton, which contain coelenterazine. However an alternative hypothesis is that the lack of dietary coelenterazine in the marina is the reason that the bioluminescence response of *Obelia longissima* growing there was found to be lower than that of colonies of *Obelia geniculata* existing in open water. As expected, all three species of *Obelia* were found to contain coelenterazine. The fact that *Obelia geniculata* and *Obelia dichotoma* had higher coelenterazine content than *Obelia longissima* supports this theory. More coelenterazine assays need to be carried out on these different species of *Obelia* to confirm if *Obelia longissima* always has a lower level of coelenterazine. Relatively low levels of coelenterazine were detected in the amphipod *Chaetogammurus marinus* and the polychaeta *Myrianida prolifer*. Young specimens of *Chaetogammurus marinus* may act as prey for *Obelia*, but once they are larger they have been observed to consume the *Obelia* hydranths. Some specimens of *Chaetogammurus marinus* were found living in cocoons on *Obelia longissima* colonies. Juveniles of these two species could be a possible dietary source of coelenterazine for *Obelia*. More extensive investigations need to be carried out to test this theory.

Oba and Kato have used deuterium labelled amino acids and electrospray ionisation-ion trap-mass spectrometry to show that the luminous copepod *Metridia pacifica* is able to synthesise coelenterazine. They have demonstrated that the copepod will synthesise coelenterazine from one molecule of L-phenylalanine and two molecules of L-tyrosine (Oba & Kato et al. 2009). Similar experiments could be carried out on amphipods to ascertain if they are capable of synthesising coelenterazine by cyclisation of amino acids. Further culturing experiments could also be conducted on *Obelia* colonies to discover if feeding colonies with coelenterazine containing copepods does maintain their bioluminescence. The question as to whether coelenterazine containing copepods act as a source of luciferin for *Obelia* colonies in the Milford Haven waterway, remains to be answered.

In *Obelia geniculata* and *Obelia longissima* colonies ratios of obelin to apoobelin were found to alter in older colonies at the end of the hydroid season, when compared to young colonies at the start of the season. In new colonies there was x 3 to 4 more obelin than apoobelin. In older colonies of *Obelia geniculata* there are close to equal levels of obelin and apoobelin. In older colonies of *Obelia longissima* there was far more apoobelin than obelin. This colony was very old and therefore loss of photoprotein may be part of the ageing process. An alternative theory is that lack of coelenterazine containing plankton in September influences the production of photoprotein. *Obelia geniculata* in June contained more apoprotein than the level detected by Campbell and Herring. They found only 10% of total photoprotein was apoobelin in their assays (Campbell & Herring, 1990). Dr Catherine Thomson has previously studied obelin apoobelin

ratios in 3 colonies of *Obelia geniculata*. Her results illustrated the opposite effect to those recorded in Pembrokeshire colonies. She found that in 2 colonies the level of obelin relative to apoobelin rose as colonies aged. In one older colony no photoprotein was detected and this may be due to its deterioration. These colonies were from samples taken in Plymouth (Thomson, 1995).

In 1981 Campbell et al extracted the photoprotein thalassicollin, from the bioluminescent radolarian *Thalassicolla*. Thalassicollin was found to rapidly emit light when stimulated with Ca^{2+} (Campbell et al. 1981). Campbell and Herring have since demonstrated that removing oxygen has "no effect on the *in vitro* Ca^{2+} stimulated chemiluminescence of obelin or thalassicollin". This contrasted with the fact that removal of oxygen completely prevented light emission from the luciferin luciferase reaction in *Vargula hilgendorfii* (Campbell & Herring, 1990).

In 1990 Campbell and Herring "reactivated apothalassicollin by addition of coelenterazine in the presence of oxygen, and removal of Ca^{2+}". They discovered that three to 20 x more photoprotein was reformed in this way, than that which was originally present. These results supported their hypothesis that in *Thalassicolla,* there is excess apoprotein and coelenterazine is the limiting factor in its bioluminescence (Campbell & Herring, 1990).

Environmental factors could influence the ratio of apoobelin to obelin; therefore more experiments need to be conducted throughout the year on different *Obelia* colonies, before clear conclusions can be drawn.

Chapter six

The effect of GFP on the wavelength of light emitted by *Obelia*

6.1 Introduction

6.1.1 The colours of marine bioluminescence

The majority of marine bioluminescence has emission maxima in the range of 450 to 530 nm. Most marine bioluminescence produces either blue or green light. "Blue light wavelength 470 to 480nm travels furthest through clear ocean water" (Herring, 2002). The hydroid *Obelia geniculata* and its medusae exhibit a bioluminescent emission peak at 508 nm i.e. green light, whereas the photoprotein obelin has a peak emission at 475 nm corresponding to blue light. The production of red bioluminescence is rare, but a few marine species i.e. *Malocosteus niger* are able to utilise it as a tool for hunting prey. In the oceans only deep sea fish of the suborder Stomiatoidea have so far been shown to exhibit red bioluminescence (Campbell & Herring, 1987). *Malocosteus niger* has been shown to contain red fluorescent material which may alter the emission peak of its bioluminescence. The visual pigments of the majority of marine species are sensitive to blue green light and therefore they can detect most bioluminescence. Very few marine eyes can detect the rare red bioluminescence (Campbell & Herring, 1987). Harvey recorded a blue fluorescence in the comb jelly *Mnemiopsis* in 1925 (Harvey, 1925). It has now been established that bioluminescent emission from ctenophores is always blue, whereas that from Cnidarians is green. The reason for this difference is that bioluminescent Cnidarians all contain GFP.

6.1.2 The effect of GFP on marine bioluminescence

Inside the photocytes of *Obelia geniculata* blue light produced from the calcium activated photoprotein obelin is thought to transfer its energy to GFP, which then emits green fluorescence. A key question that remains to be answered is what the selective advantage of GFP and or other fluors? The reason that Cnidarians have evolved to produce green bioluminescence rather than blue is still being investigated. Morin has suggested that the early work of Nicol on pelagic coelenterates may contribute to solving this question. Nicol suggested that marine species in relatively shallow waters produce green bioluminescence whereas those at greater depth (less light) produce blue light (Nicol, 1958: Morin, 1974). More recent research by Steven Haddock supports the hypothesis of Nicol (Haddock et al. 2010). "Bioluminescence is not essential for the survival or reproduction of luminous species." A K Campbell has predicted that the selective advantage of marine fluors could be linked to temperature control. "The selective advantage of fluorescence, including GFP and colours in corals and anemones, in many coelenterates is likely to be related to temperature. This is because fluors absorb light energy and reemit it without allowing the light energy to be converted to heat" (Campbell, 2012).

In the sea pansy *Renilla* there has been confusion as to whether GFP amplifies the quantum yield of light produced by the organism, i.e. produces a brighter light. William Ward has now established that in *Renilla* GFP binds the luciferase increasing the quantum yield of the reaction by a factor of three. Ward and Cormier have showed that *Renilla* transfers energy from a luciferase-oxyluciferin complex by a "radiation less energy transfer mechanism" to its GFP (Ward &

Cormier, 1979). An important equation for this dipole-dipole resonance energy transfer was published by Forster in 1948 (Forster, 1948). This predicted that at higher concentrations of GFP (>100 mM) molecules of GFP and luciferase must be less than 100 Angstroms apart. Ward has now established that in *Renilla* GFP binds the luciferase increasing the quantum yield by a factor of 3. However it does not bind to aequorin in *Aequorea* and there is no increase in quantum yield (Ward, 2006). Waud et al have discovered that GFP will increase quantum yield from aequorin when the photoprotein and GFP are engineered to be linked together (Waud et al. 2001).

 GFP is not thought to increase quantum yield in *Obelia geniculata* (Shimomura, 2006).The cyclised chromophore of GFP is made via the modification of 3 amino acids within its polypeptide structure. These have been shown to be serine, tyrosine (dehydrogenated) and glycine. GFP consists of 238 amino acids which form an 11 stranded barrel of beta sheets that surround the alpha-helical heptapeptide of its fluorescent centre. The chromophore is covalently bound to the centre of the beta barrel. The elucidation of GFP's structure has occurred due to the work of many different research groups. Davenport and Nicol found a fluorescent substance in *Aequorea* in 1955 (Davenport & Nicol, 1955). In 1962 Shimomura working on *Aequorea* from Friday Harbor at Princeton University, observed a "green protein" which fluoresced within his aequorin extracts. He noted that the protein produced no luminescent reaction (Shimomura et al. 1962). In 1969 this green protein was named green fluorescent protein (GFP) by Hastings and Morin (Hastings & Morin, 1969; Chalfie & Kain, 1998). Morise et al discovered 3 isoforms of GFP in *Aequorea* which he partially characterised

(Morise et al. 1974). In 1979 Shimomura reported that GFP's molecular weight was approximately 27,000 and that it was a relatively stable protein. Prasher et al cloned and sequenced the cDNA for GFP from *Aequorea.* They found its molecular weight to be 26,888 and its emission max was 508 nm (Prasher et al. 1992).This agreed with the emission maxima of 508 to 509 nm which Shimomura recorded for bioluminescence from live specimens of *Aequorea*. The chromophore structure of GFP was elucidated by Cody et al in 1993 (Cody et al. 1993). Ward has demonstrated that GFP is a monomer at concentrations below 0.5 mg/ml, but exists as a dimer at higher concentrations. This does affect its absorption spectra at higher concentrations. *Aequorea's* photocytes do contain relatively high concentrations of GFP and aequorin. Cutler and Ward have simulated the *in vivo* bioluminescence of *Aequorea* by mixing high concentrations of GFP and aequorin together in a capillary tube. The resulting green light emitted does suggest that radiation less energy transfer between the two proteins can occur (Cutler & Ward, 1997). For such energy transfer to happen GFP and the photoprotein must be less than 100 Angstroms apart. The absorption spectra of the GFP must also overlap with the emission spectra of the photoprotein (Morin, 1974; Shimomura, 2006). Previously Ward and Cormier showed that *Renilla* transfers energy from a luciferase-oxyluciferin complex by a "radiation less energy transfer mechanism" to its GFP. Whether this same mechanism is utilised in *Aequorea* or *Obelia geniculata* is a matter of debate (Morise et al. 1974; Ward, 1979). "Radiative energy transfer may occur in *Aequorea* i.e. direct transfer of a photon from donor to acceptor*"*. The GFP of *Aequorea* and *Renilla* is not identical as they have different absorption spectra and molecular weights. Ward has hypothesised that

the chromophores of the two GFP's must be identical and that the absorption spectra differences are due to the different apoprotein environments (Ward & Cormier, 1979; Ward, 1980). Shimomura has investigated the similarities in structure of the GFP chromophore and coelenterazine itself. When the chromophore of GFP was isolated it was found to be an imidazolopyrazine.

"It is intriguing that the structure of the GFP chromophore is part of the structure of coelenterazine" (Shimomura, 2006). Both molecules can be formed by the cyclisation of amino acid trimers via a very similar mechanism.

6.1.3 Photobleaching of GFP

When demonstrating the existence of wild type GFP in *Obelia* to participants of the hydrozoan society's workshop at the MBA Plymouth in 2007, and at the EMBO microscopy workshop at Plymouth in 2010, organised by Prof Brad Amos, we noticed very little loss of the fluorescence of hydroids left under continuous excitation of a Hg UV lamp for over 3 hours. This indicated that wild type GFP in *Obelia* did not readily photobleach. Yet it is well known that many fluors undergo rapid photobleaching (Song et al. 1995; Sheng & Yunus, 2002; Eggeling et al. 2005). Conversely, we demonstrated that genetically engineered GFP in *E. coli* bleached within a few minutes. An initial experiment on *Obelia geniculata* showed that there was no significant photobleaching of its GFP over a period of 83 minutes. This warranted further investigation.
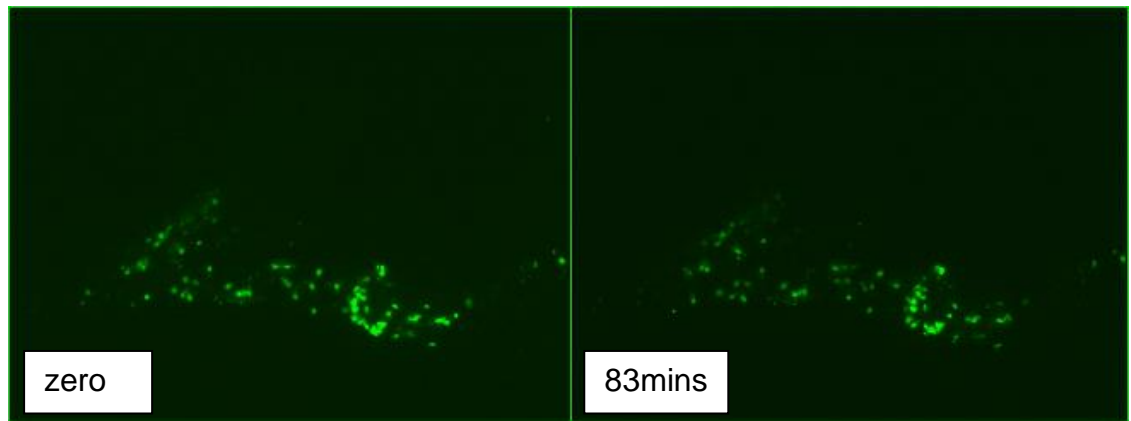
Fig.6.1. Fluorescent images of GFP in *Obelia geniculata* recorded using the Zeiss Ultraphot fluorescent microscope, at magnification x10. UV lamp set on max 4 intensity giving120 watts 488nm. Left hand image is fluorescence at time zero and right hand image shows fluorescence after 83 min.

Genetically engineered fluors such as GFP are now extensively used as reporter genes. The phenomenon of photobleaching means that continuous fluorescent measurements of cells expressing these fluors cannot be taken. Photobleaching also produces free radicals which are poisonous to the cells. The photobleaching effect on dyes has been known for some time e.g. the photo bleaching of methylene blue. The dye methylene blue (MB) when linked to a gelatin matrix is known to photo bleach under UV light. The dye becomes photo reduced to a colourless form. Carreto et al (Carreto et al. 2001) have proposed that due to absorption of UV light "the MB molecule is photo reduced in a photochemical reaction resulting in a production of leuco (colourless) form of dye molecules" (Carreto et al. 2001). This reaction can be exploited in holography (Pradeep et al. 2000).

6.1.4 Photobleaching in *Obelia*.

The key question to be answered is: what protects GFP in *Obelia* from photobleaching? The hypothesis was that within the hydroid *Obelia* there is a

molecular mechanism protecting its GFP from photobleaching. The mechanism is likely to involve coelenterazine, which is known to be an antioxidant (Devillers et al. 2001; Arrault et al. 2003; de Wergifosse et al. 2004; Saleh & Plieth, 2010). Rees et al have described coelenterazine "as the marine equivalent of vitamin C" due to its antioxidant properties (Rees et al. 1998).

The aim of the experiments in this chapter was to confirm if the different species of *Obelia* found in Pembrokeshire all emit bioluminescence and fluorescence at the same wavelengths, and to confirm that any green emission was due to the presence of GFP. Fluorescence in a number of non luminous species would also be assessed as a comparison. A second aim was to quantify photobleaching of GFP in 2 species of *Obelia* hydroids and compare it with photobleaching of genetically engineered GFP in *E. coli*. The GFP in *E. coli* was EGFP, the S65T variant, which expressed a brighter fluorescence within the bacterial cells. The structure of the *Obelia* photocytes was also investigated. Fluorescent indicators in cells photobleach, but a key question is "does *wild type* GFP in Obelia photobleach?"

The experimental strategy included:

An investigation to confirm that GFP was responsible for fluorescence in luminous hydroids. Some non luminous species were also investigated. This utilised the following methods:

- Confocal fluorescent microscopy to measure the fluorescent maxima for *Obelia* species.

- Dual wavelength chemiluminometry to calculate the ratio of blue to green light in the bioluminescent emissions of *Obelia* species.

- Photek analysis software to analyse fluorescent images of *Obelia* species to calculate the ratio of blue to green light in their bioluminescent emissions.

- Confocal microscopy to investigate the structure of the photocytes of *Obelia*.

The strategy was also to compare the loss of fluorescence in Obelia species with loss of fluorescence in EGFP, by conducting experiments on photobleaching.

- Photobleaching of GFP in *Obelia* and in *E. coli* was assessed under two different UV lamps of two different fluorescent microscopes. To compare loss of fluorescence in *Obelia* species with loss of fluorescence in EGFP.

The Photek imaging system was also used to measure the spectrum of the bioluminescence of the photoprotein obelin.

6.2 Methods and materials.

6.2.1 Confocal microscopy

In 1955 the first confocal microscope was invented by Marvin Minsky. This microscope produces high resolution images of a specimen in two or three dimensions. Modern confocal microscopes utilise the key components of pinhole apertures, scanning mirrors and a laser light source to remove the majority of "light from the specimen which is not from the microscope focal plane" (Semwogerere & Weeks 2005). This technique can increase the contrast within a

specimen image as well as reducing image blurring. Due to the fact that a high percentage of the light from the sample is excluded by the aperture, light intensity is low. Therefore long exposures are needed as the microscope scans one point of the specimen at a time i.e." point by point illumination". As only one point of the sample is being illuminated at a time the detector and image analyser must construct the image one pixel at a time. Each image thus constructed "represents a thin cross- section of the specimen". A series of these cross-section images can be reconstructed into a 3D image (Semwogerere & Weeks, 2005). Confocal microscopy was used to produce a 3D image of a photocyte in *Obelia.*

6.2.2 Confocal fluorescent microscopy

This was used to measure the fluorescent emission maxima for two species of *Obelia.* The confocal microscope in the school of Pharmacy was used which is a Leica TCS SP5 with a Spectra Physics Stabilite 2017 UV laser. The machine was focused onto the GFP in the hydroid's photocyte. The GFP was excited by shining light on to it at a wavelength of 488 nm. The machine recorded the intensity of fluorescence emitted over a range of wavelengths. There were 5 nm separations between these wavelengths. The emission maxima is the wavelength at which most of the fluorescence is emitted.

6.2. 3 Photek spectra

The spectrum of the bioluminescent light produced by the photoprotein obelin was measured using the Photek imaging system. This has already been described in Section 2.2.2.

6.2 .4 Dual wavelength chemiluminometer

The chemiluminometer has already been described in 2.2.1.To calculate the ratio of blue to green light in bioluminescent emissions; a dual wavelength chemiluminometer was used. This has two photomultiplier tubes; set up to measure blue and green light respectively. 20nm interference filters were used. Photon counts from blue light (475 nm) were recorded on channel one, whereas photon counts from green light (525 nm) were recorded on channel two. From this data the ratio of blue to green light was calculated in an Excel spread sheet.
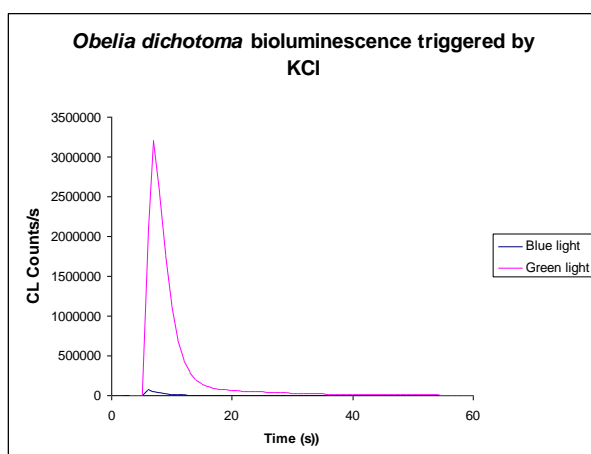
Fig.6.2. An example of dual wavelength chemiluminometer data. Data obtained from the bioluminescent emission of *Obelia dichotoma*. *Obelia dichotoma* was stimulated with 1ml of 0.5M KCl. As expected a high % of the bioluminescence produced was green light rather than blue.

6.2. 5 Photek analysis of RGB from fluorescent images

Images of the fluorescence produced by the GFP in different hydroid species including *Obelia's* medusa were captured using the Hund H500 fluorescent microscope linked with the Sentech camera. The details of this technique have already been discussed in 3.2.8. Photek analysis software was then utilised to measure the red, green and blue components of this fluorescence. Ten odd area set

(OAS) circles are drawn on the most fluorescent parts of the image from the specimen (refer to Fig.2.8.). The software then calculates the photons produced by that OAS and splits them into photon counts for each wavelength of light. This produces photon counts for the red, blue and green components of the fluorescence. An excel spread sheet was used to calculate the ratio of green to red light, green to blue light and red to blue light.

6.2. 6 Photo bleaching

The photobleaching of GFP in *Obelia geniculata* was recorded in two experiments. An initial experiment recorded fluorescence at time zero and compared it with the fluorescence present after the hydroid had been under the UV lamp for 83 min. The Zeiss Ultraphot fluorescent microscope was utilised for this at magnification x10.The UV lamp was on the maximum intensity setting of 4 and produced 120watt at a wavelength of 488 nm. On each fluorescent image 10 odd area sets (OAS) were created, where high fluorescence occurred, using the Photek analysis software. The intensity of fluorescence in each of these areas was extracted at time zero. The same OAS areas were then moved to non fluorescent areas of the image and intensities were again extracted. These measurements form the background and are then subtracted from the fluorescence intensities (refer to Fig.2.8.). The same process was repeated at 83min. The fluorescence intensities minus background calculated for each OAS at 83 min, were then expressed as a percentage of the original fluorescence intensity calculated for that area at time zero and was referred to as the Odd area profile (OAP). A second more rigorous experiment was then conducted to produce a time course of fluorescence in *Obelia*

geniculata over 6 h. A fluorescent image was recorded every hour for 6 hours, again using the Zeiss Ultraphot on maximum intensity refer to Fig. 6.16.

6.2.7 Calculating half-life $t_{1/2}$ for GFP bleaching.

Fluorescence was plotted on a log plot against time. The slope of this graph was used to determine the half-life $t_{1/2}$ for GFP bleaching.

6.2.8 *E. coli*

Genetically engineered *E. coli* expressing GFP, were a kind gift from Dr David Dowling, IT Carlow Eire. They were grown up on plates and then a small amount of the *E. coli* was placed on a slide so that it could be viewed under the fluorescent microscope.

6.3 Results

6.3.1 Results from confocal fluorescent microscopy
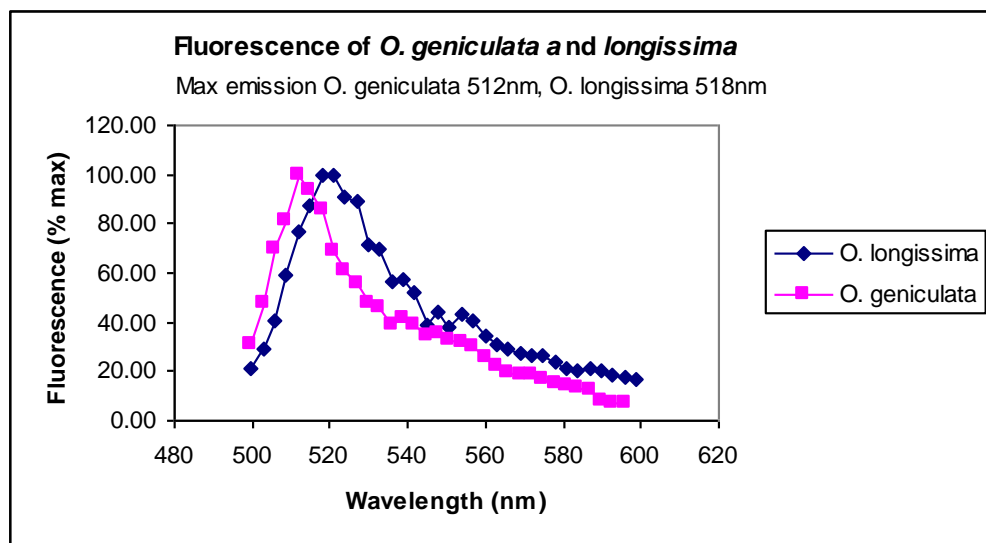
Fig.6.3.Fluorescent emission maxima for *Obelia geniculate* and *Obelia longissima.* The fluorescent emission maximum was 512 nm for *Obelia geniculate*, which is very green for this species. The fluorescent emission maximum was 518 nm for *Obelia longissima.*

The results suggest that the GFP in these two species is not identical. These differences could be due to environmental factors. Morin and Hastings recorded λ max of 508nm for both species.

6.3.2 Results from dual wavelength chemiluminometer
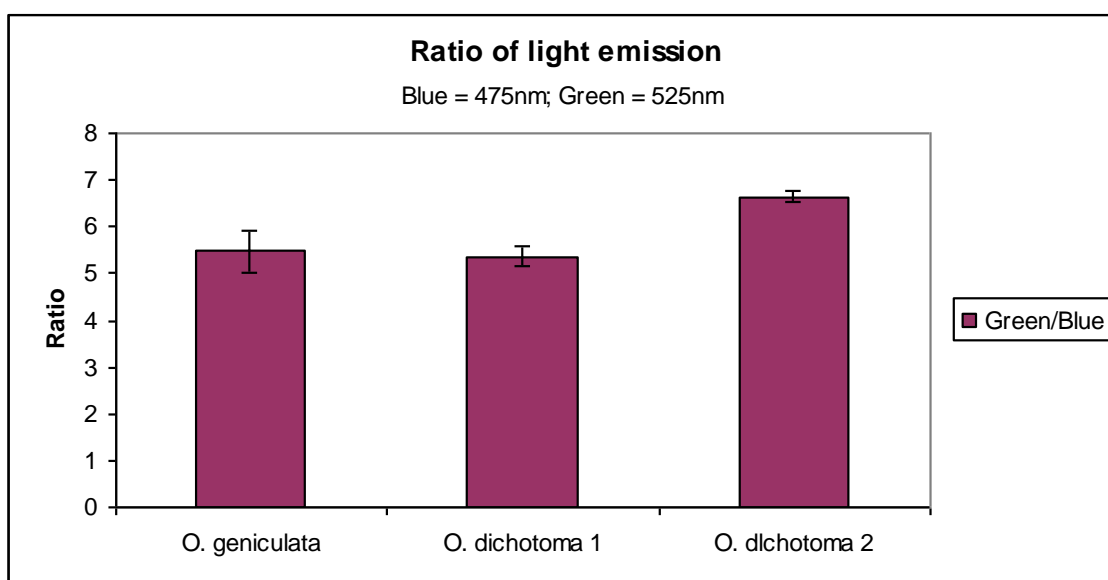
Fig.6.4. Dual wavelength chemiluminometer, the ratio of green to blue light emission was calculated for one colony of *Obelia geniculata* and two colonies of *Obelia dichotoma.* Bioluminescence of hydroids was stimulated by adding 1ml of 0.5M KCl. Results represent the mean of 6 results ± SEM.

The ratio was similar for all 3 colonies. As expected in all specimens, the majority of light emitted is green with only a small blue component. There was a small difference in the ratio for *Obelia geniculata* and *Obelia dichotoma.* A difference was also found in the ratios for the two different colonies of the same species *Obelia dichotoma.* These differences might be caused by environmental factors.
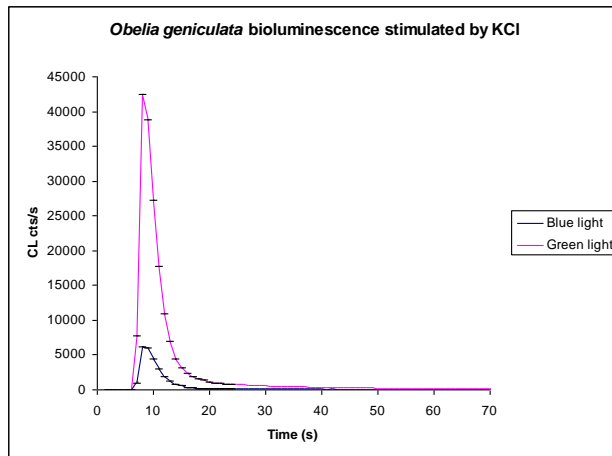
Fig.6.5. Results from the dual wavelength chemiluminometer. *Obelia geniculata* was stimulated with 1ml of 0.5M KCl. As expected a high % of the bioluminescence produced was green light rather than blue. The results show the mean photon counts from 6 hydroids ± SEM.

6.3.3 Results of colour ratios for fluorescence in hydrozoans
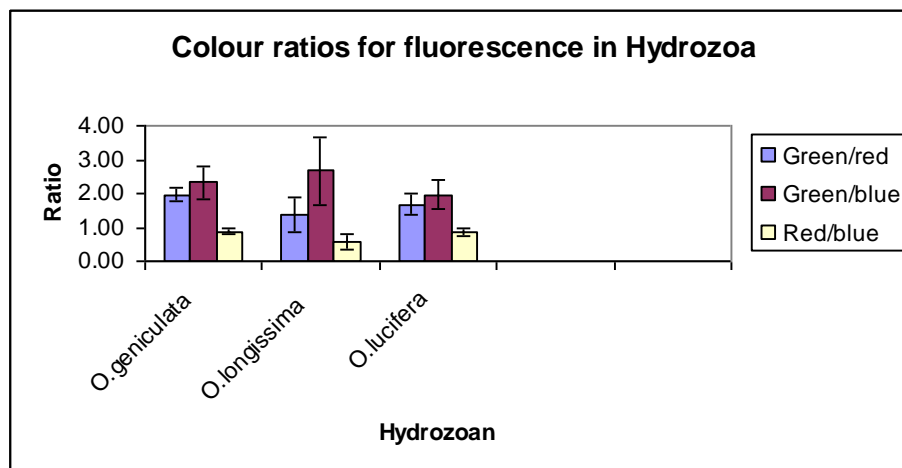
Fig.6.7. Colour ratios for fluorescence in hydrozoans. Photek analysis software was utilised to calculate colour ratios from images of the fluorescence emitted by *Obelia geniculata, Obelia longissima* and the medusa *Obelia lucifera.* Each result represents the mean of 10 measurements ± SEM.

In all three species green fluorescence was the largest component of overall fluorescence, and there was only a small amount of red fluorescence. These results illustrate that the fluorescence is produced by GFP and is not due to another fluor. Comparisons were made between these results and fluorescence produced by a

siphonophore *Muggiaea atlantica*, a non bioluminescent medusae *Sarsia eximia* and *Clytia*.
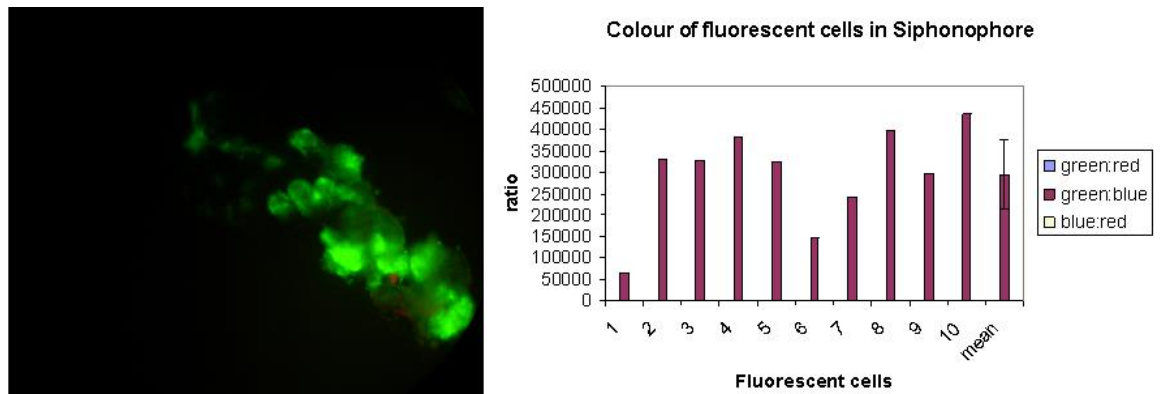
Fig.6.8. Image of fluorescence in the siphonophore *Muggiaea atlantica*, taken with the Hund fluorescent microscope x 100 and, the Sentech camera. The fluorescence was analysed as a comparison using the Photek analysis software. Fluorescence emitted from the siphonophore was found to have very high levels of green fluorescence, no blue light component, and only a very low level of red light. This is most likely due to the presence of a fluor other than GFP.

This fluorescence from the siphonophore was found to have no blue light component, only green and red light. This illustrates that not all marine fluorescence is due to GFP.
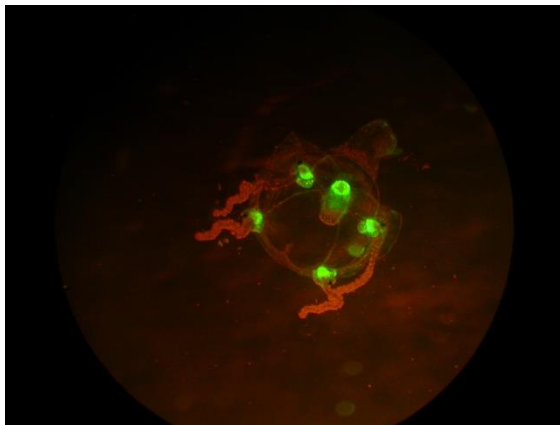
Fig.6.9. Fluorescence in the medusa *Sarsia eximia*. The image of this medusa was taken with the Hund fluorescent microscope using x 100 magnification and the Sentech camera. The fluorescence was analysed as a comparison using the Photek analysis software. Fluorescence emitted from the medusa was found to have no blue light component.
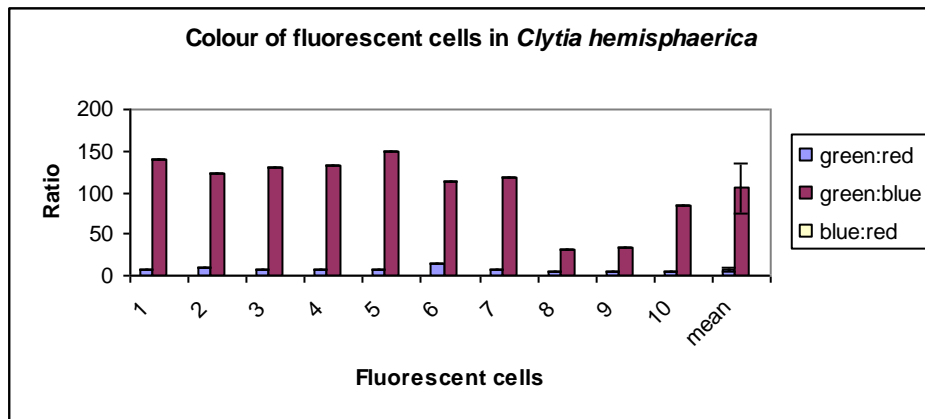
Fig.6.10. Photek analysis of fluorescence emitted from *Clytia hemisphaerica*. This had a very high green light component.
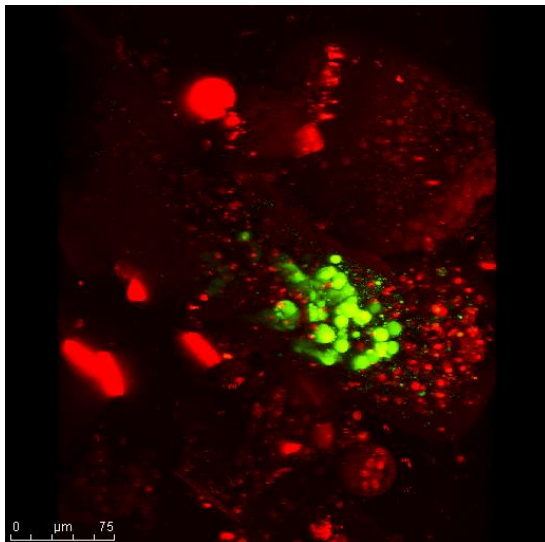
Fig.6.11. Confocal image of a photocyte of *Obelia longissima*. The photocyte in this species is situated *in* the annulations beneath a hydranth. This image suggests that the photocyte is particulate in nature.

An attempt was made to measure the spectra of the photoprotein obelin extracted from *Obelia geniculata* using the Photek imaging system. The diffraction grating was set at 500 nm. The spectrum obtained was not of sufficient quality to be presented. This experiment could be repeated to confirm that obelin produces blue

light, whereas the hydroid produces green light. It is the GFP inside *Obelia geniculata's* photocytes which lengthens the wavelength of emission.

6.3.4 Photobleaching of GFP in *Obelia geniculata*

In order to examine whether GFP in Obelia geniculata photobleaches it was studied under the Xcite microscope for up to 83 min. Results showed after 83 min the mean GFP fluorescence is 81.7% ± 5.2%. The range of results is 59.4% to 117%.
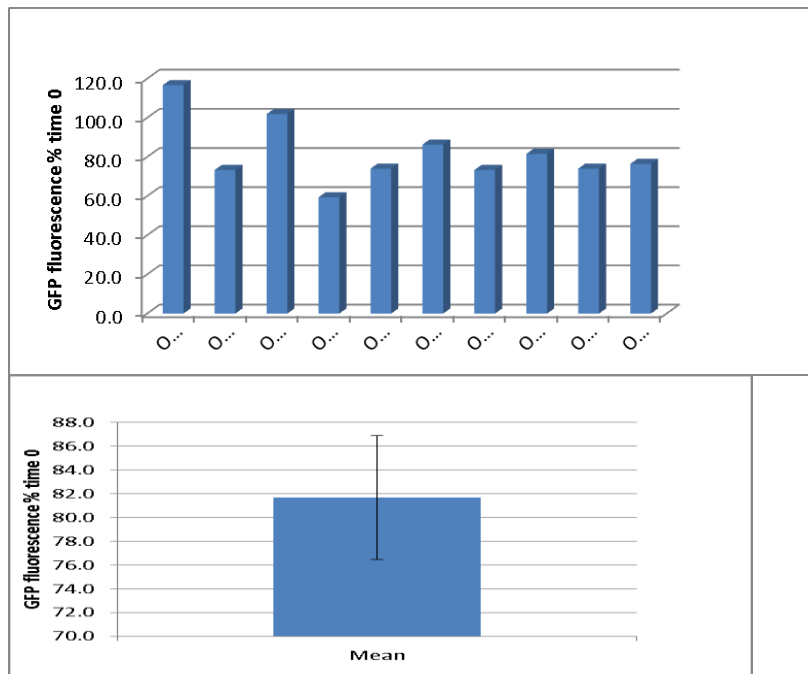
Fig.6.13. GFP fluorescence in *Obelia geniculata*. The fluorescence at each odd area profile (OAP) was expressed as a % of that originally present at time zero. This illustrated that at OAP1 fluorescence had increased, whereas at OAP 2 it has remained constant. At all other OAP's it has reduced. The range of results was 59.4% to 117%.The mean GFP fluorescence is 81.7% ± 5.2% after 83 min demonstrating very little loss of fluorescence i.e. a very low level of photobleaching.
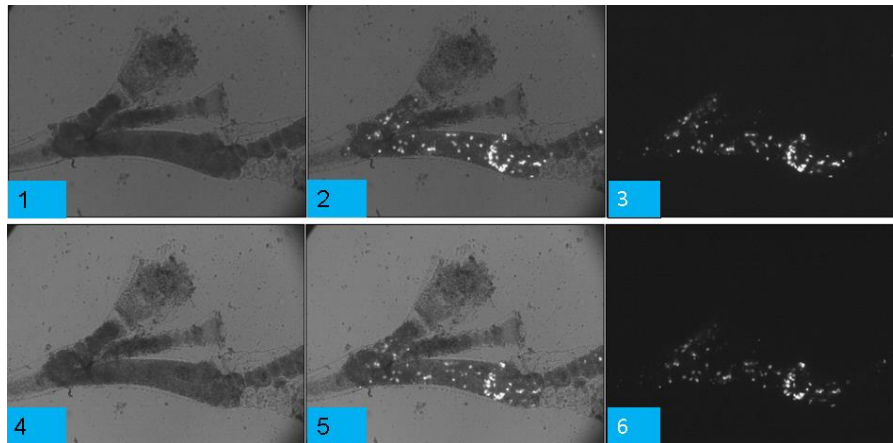
Fig.6.14. Black and white images of *Obelia geniculata*. Fluorescence recorded using the Zeiss Ultraphot fluorescent microscope. The Magnification was x10. The UV lamp was set on the maximum intensity of four ie 120 watt. Images 1 to 3 from left to right are the bright field, bright field and fluorescence, and fluorescence alone recorded at time zero. Images 4 to 6 from left to right are the bright field, bright field and fluorescence, and fluorescence alone recorded at time 83 min.

This illustrates that after 83 minutes there is very little loss of fluorescence in the GFP of *Obelia geniculata*. A longer time course was then recorded for photobleaching in *Obelia geniculata*. Fluorescence of GFP was recorded every hour for six hrs, using the Xcite microscope. The $t_{1/2}$ for GFP bleaching in *Obelia geniculata* was then calculated.
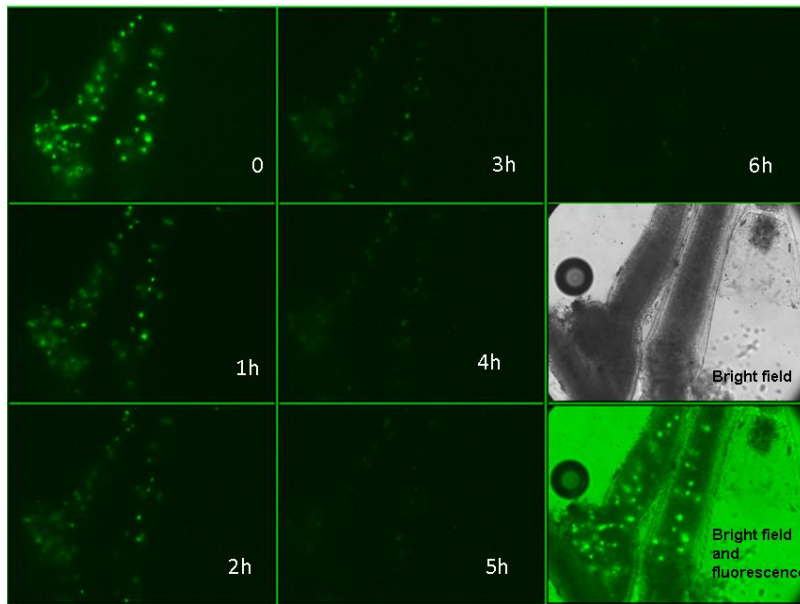
Fig.6.15. A time course of fluorescence in *Obelia geniculata*. Fluorescence was recorded every hour for 6hrs. Top left image is time zero. Under the maximum fluorescence of Xcite 120 watt lamp at 488 nm. Zeiss microscope magnification was x10. The $t_{1/2}$ for GFP bleaching in *Obelia geniculata* under this lamp is 60.3 ± 3.1 min.
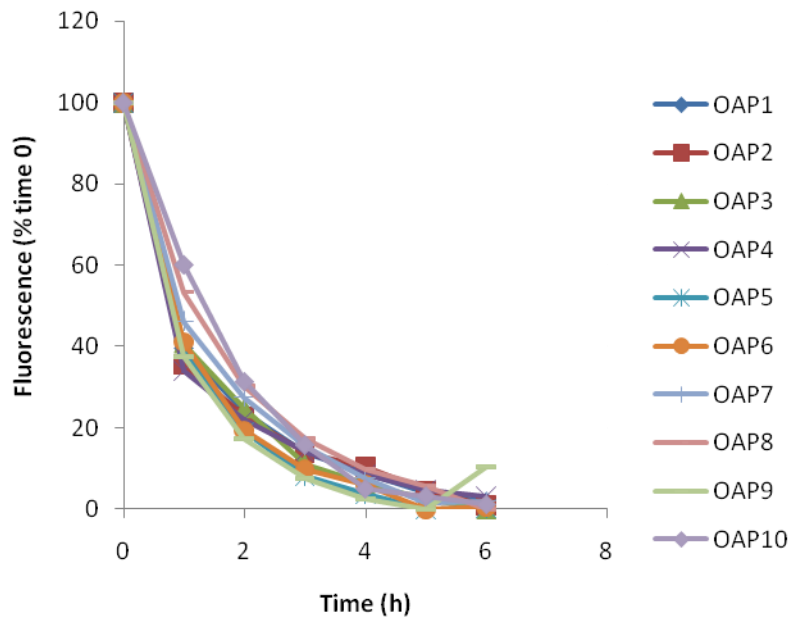
Fig.6.16. Ten Odd area profiles (OAP) for GFP bleaching in *Obelia geniculata*. The Xcite lamp was on the maximum setting of four ie120 watts at 488 nm. Zeiss microscope magnification was x10. The $t_{1/2}$ for GFP bleaching in *Obelia geniculata* under this lamp was 60.3 ± 3.1 min. Range of results 45.7 to 74.0 min.

209
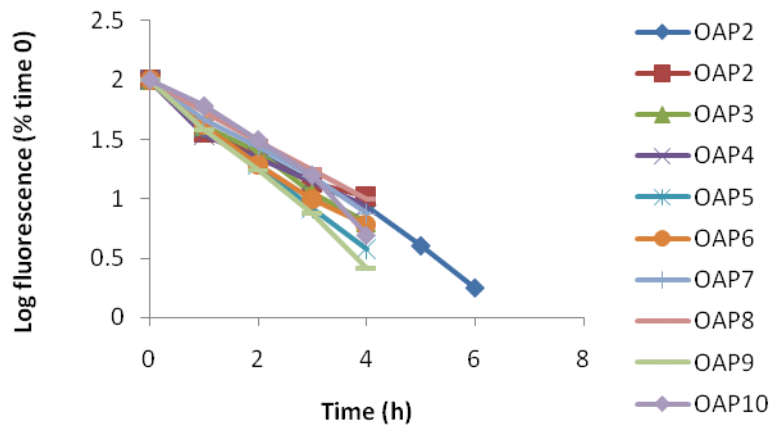
Fig.6.17. illustrates the log plot of % fluorescence over time in hours. Slope of this graph was used to determine the fluorescence half-life of the *wild type* GFP in Obelia geniculata's photocytes. *Obelia geniculata* 0 to 6hrs under maximum fluorescence of Xcite lamp, 120watt, 488 nm. Photobleaching is linear which suggests the rate of the reaction is first order.
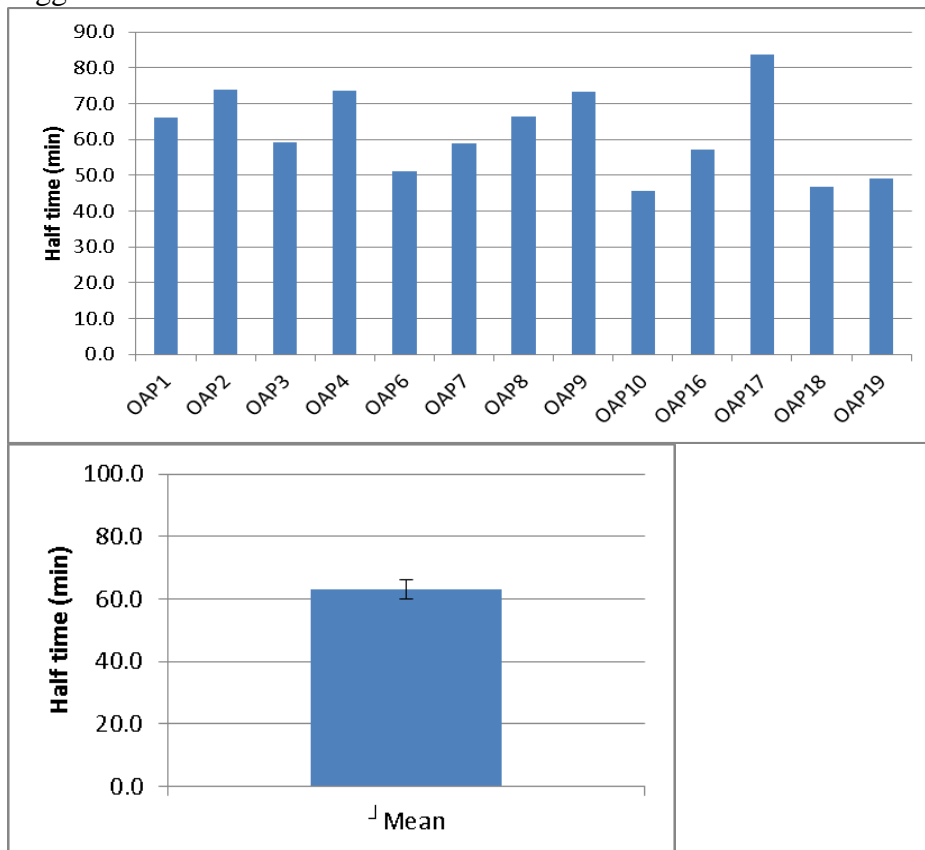
Fig.6.18. *Obelia geniculata* 0 to 6hrs under maximum fluorescence of Xcite lamp, 120watt 488nm. $t_{1/2}$ of photo bleaching = 63.0 ± 3.1min. Range 45.7 to 83.6.

6.3.5 Results photo bleaching of GFP in *Obelia dichotoma.*

To determine whether, GFP in *Obelia dichotoma* photobleaches, an initial trial experiment was conducted over two hours. *Obelia dichotoma* was obtained from Milford Haven marina and placed under the Xcite lamp 120 watt. Images of fluorescence were recorded every hour (refer to Fig.6.19).
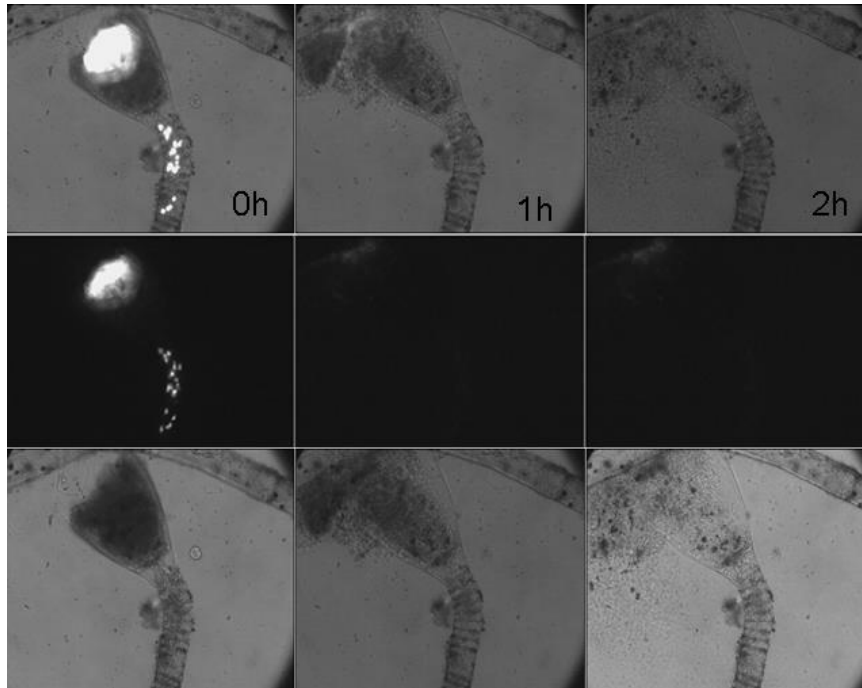
Fig.6.19. Photobleaching of GFP in *Obelia dichotoma*. Photobleaching of GFP in photocytes, within the annulations beneath a hydranth. Top row of images left to right show fluorescence of GFP in *Obelia dichotoma* annulations. Under Xcite lamp 120watt 488nm, at 0, 1 and 2h. Second row images are fluorescence and bright field combined. Bottom row represents bright field only. Zeiss fluorescent microscope magnification was x20. Range at 1h = 8.4 to 19.6%.

Bleaching of GFP in Obelia dichotoma
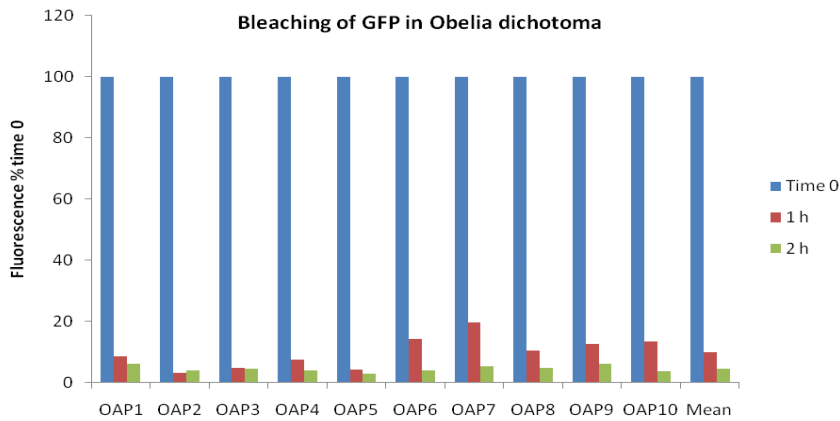
Fig 6.20 Photobleaching of GFP in *Obelia dichotoma*. Illustrating how fluorescence rapidly diminished in 10 areas of GFP over 2h. Xcite lamp set at 120watt 488nm, Zeiss fluorescent microscope magnification x20. Range at 1h = 8.4 to 19.6%.

An additional experiment was conducted observing the GFP in the photocytes in the stem of *Obelia dichotoma* to ascertain if the rate of photobleaching is the same in all photocytes regardless of position.

Bleaching of GFP in the stem of O. dichotoma
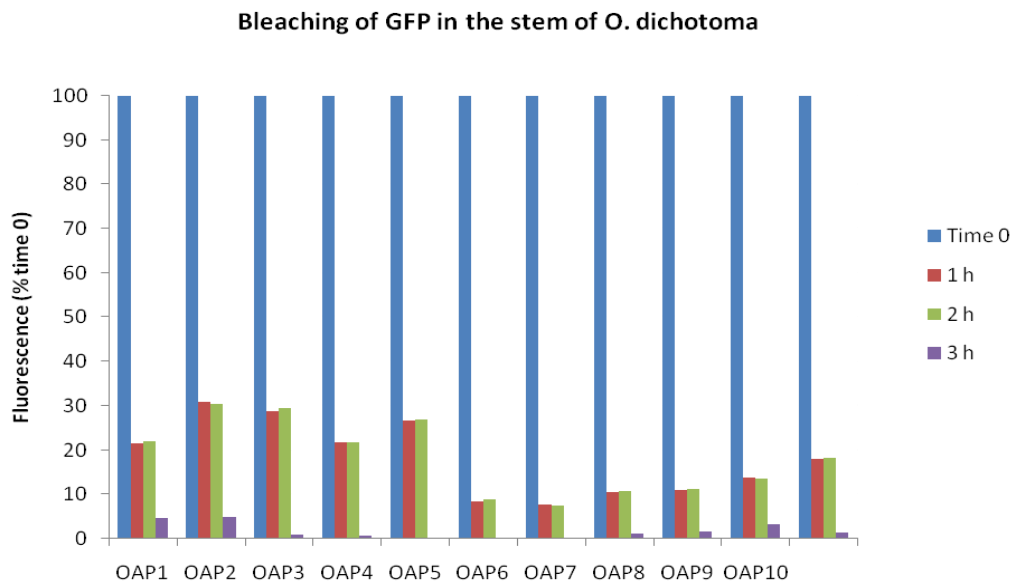
Fig.6.21. Photobleaching of GFP in the stem of *Obelia dichotoma*. Illustrating how fluorescence rapidly diminished in 10 areas of GFP over 3h. The rate of photobleaching was lower than that observed in GFP in the annulations. Xcite lamp set on 120watt 488nm, Zeiss fluorescent microscope magnification x20. Range at 1h = 8.4 to 19.6%.

212
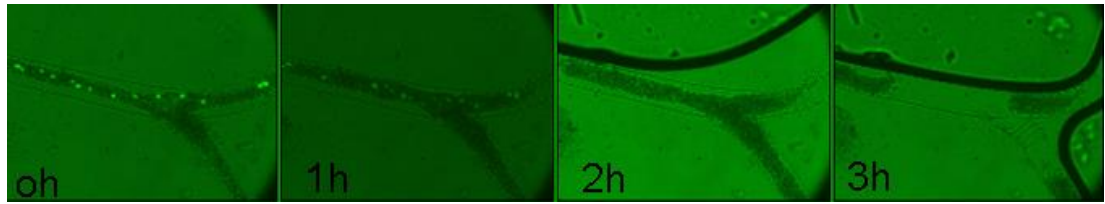
Fig.6.22. Photobleaching of GFP in the stem of *Obelia dichotoma*. Left to right shows fluorescence and bright field combined for 0, 1, 2, and 3h.Illustrating how fluorescence rapidly diminished in 10 areas of GFP over 3h. The rate of photobleaching was lower than that observed in GFP in the annulations of *Obelia dichotoma*. The Xcite lamp was set on 120watt 488nm, Zeiss fluorescent microscope magnification x40.

This rapid photobleaching of GFP in *Obelia dichotoma* was worthy of further

investigation. The effect of reducing the strength of the UV was studied by

reducing the setting of the Xcite lamp by 50% i.e. setting 2 = 60 watts.

Photobleaching was also studied under the Hund microscope Hg 100W lamp x10

objective from 0 to 6hrs.
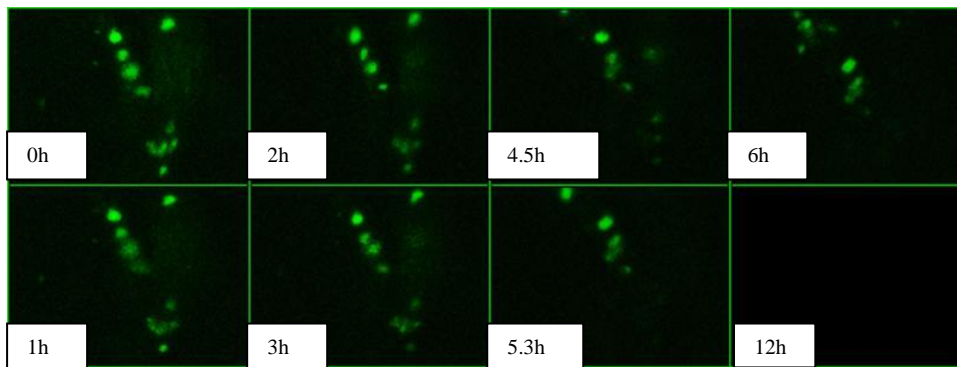
Fig 6.23 Photo bleaching of *Obelia dichotoma* (48h old specimen from Milford Haven marina), 0 to 6 hrs under Hg 100W lamp x10 objective Hund fluorescent microscope linked with Sentech camera. Mean $t_{1/2}$ of photo bleaching = 5.9 ± 0.8 h. Range 2.7 to 9.1h.This illustrates that even after 6 hrs of continuous excitation the GFP in *Obelia dichotoma* exhibits a very low level of photobleaching, if the power of the lamp is reduced.

213
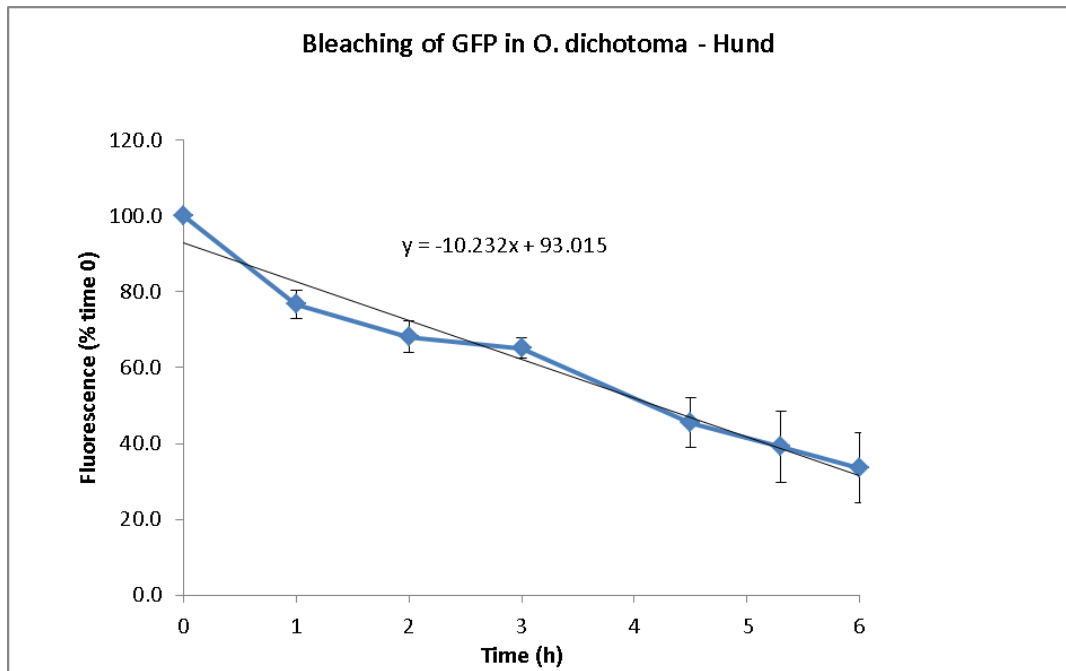
Fig 6.24. Photobleaching of *Obelia dichotoma* % fluorescence over time under Hg 100W lamp x10 objective Hund fluorescent microscope. A trendline was drawn to the curve. Mean $t_{1/2}$ of photo bleaching = 5.9 ± 0.8 h. Range 2.7 to 9.1 h. This represents a very low level of photobleaching. Reducing the strength of the lamp lowers the level of photobleaching.
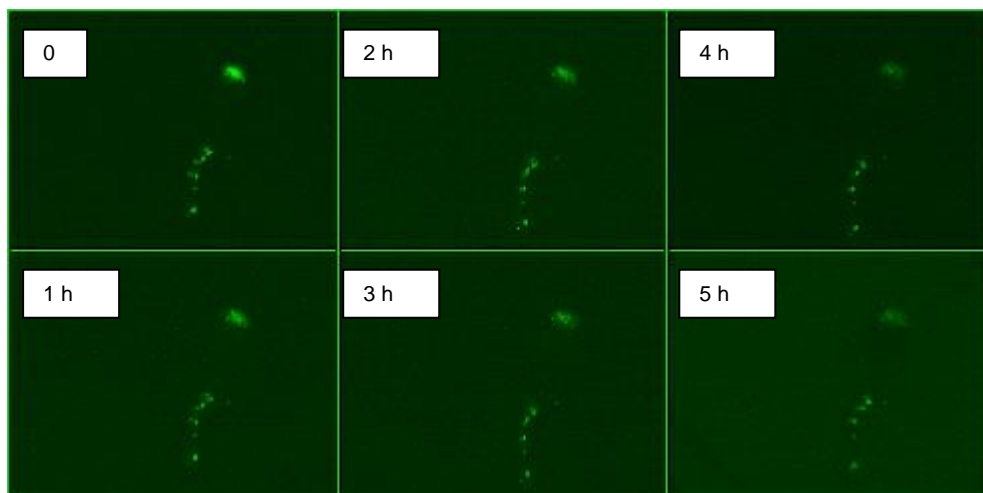
Fig.6.27. Photo bleaching of *Obelia dichotoma* (3 day old specimen from Milford Haven marina). Recorded under Xcite lamp setting 2, producing 60 watt, *using* Zeiss Xcite microscope. Mean t $_{1/2}$ of photobleaching = 6.6 ± 1.3 h. Range 3.1 to 15 h.
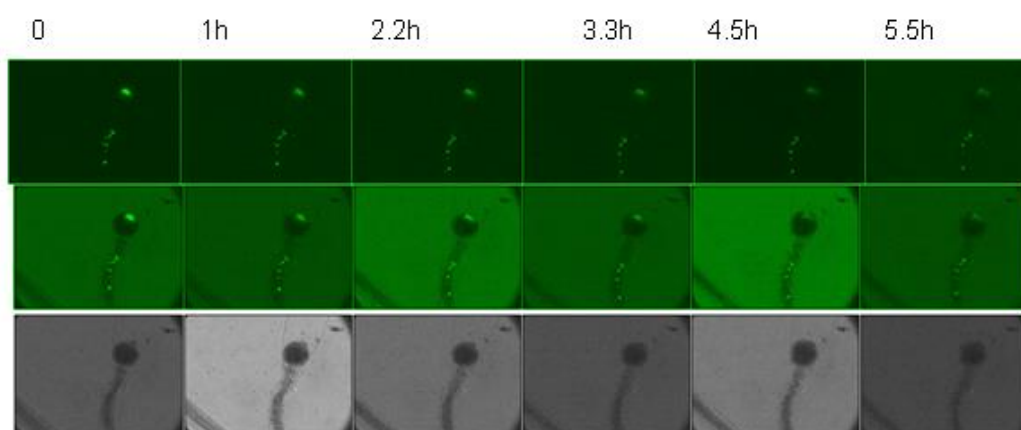
Fig.6.28. Photo bleaching of *Obelia dichotoma* as in Fig 6.13. Images recorded every hr. Top row left to right represent fluorescence over time. Middle row represents fluorescence and bright field images combined. Bottom row represents bright field images only. A very low level of photobleaching was recorded in this species, when placed under a 6owatt lamp.
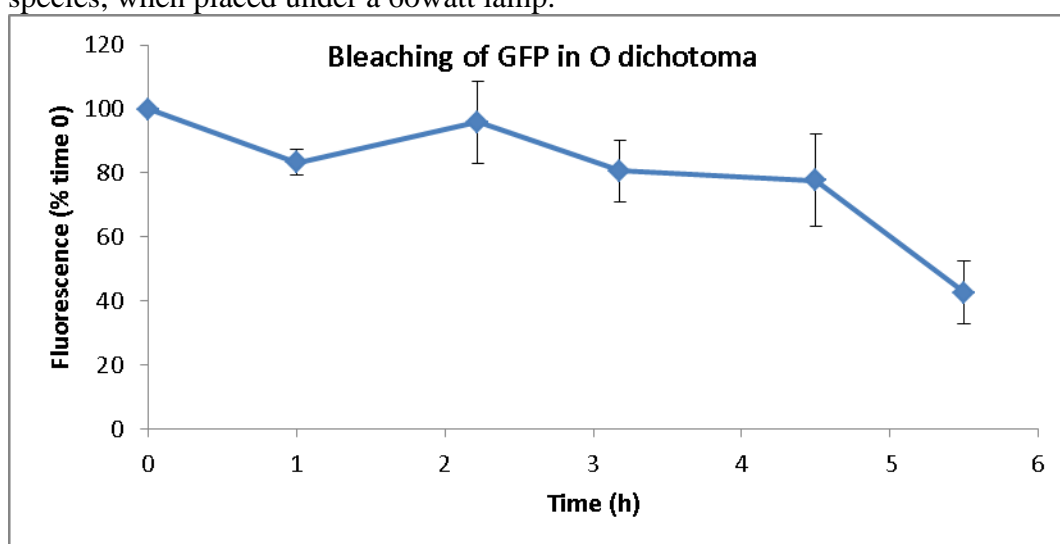
Fig.6.29. Percentage fluorescence over time of *GFP* in *Obelia dichotoma*. Measured under Zeiss Xcite lamp 60 watt. Mean $t_{1/2}$ of photobleaching = 6.6 ± 1.3h. Range 3.1 to 15 h.

Half-life for GFP bleaching in *Obelia dichotoma* is slightly lower when using the Hund Hg lamp, although it was producing a higher wattage. Both sets of data show a close to linear reaction which suggests that in *Obelia dichotoma* photobleaching has a first order rate of reaction.

215

Species	Lamp	$t_{1/2}$
GFP in Obelia dichotoma	Hund Hg 100W lamp	5.9±0.8h
GFP in Obelia dichotoma	Excite lamp Setting 2 60watt	6.6 ±1.3hrs

Table 6.1 A comparison of mean $t_{1/2}$ for photobleaching of GFP in *Obelia dichotoma* under two different fluorescent lamps. Photobleaching occurred at a very low rate, compared to the trial experiment. Reducing the power of the lamp significantly reduces the rate of photobleaching.
A T-test comparing the two $t_{1/2}$ values gave p as <0.001 .This illustrates that the half lives are highly significantly different.

Results for photo bleaching of EGFP in *E.coli*

An initial experiment produced a time course over 17mins for the bleaching of

EGFP in *E.coli* .A fluorescent image was recorded every minute. Bleaching was

recorded using the Zeiss microscope with excite lamp on the maximum 4 setting.

To completely quantify photobleaching in EGFP a time course was then recorded

over 10hrs.
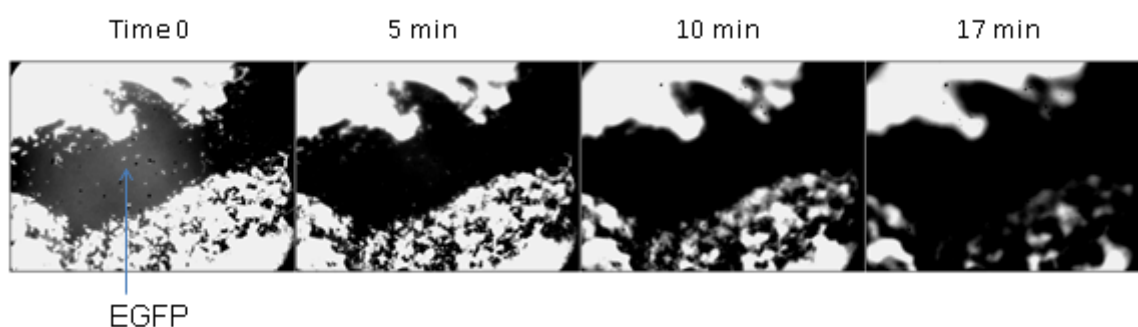
Fig.6.32. Bleaching of EGFP (Bright field image) in *E. Coli* recorded using the

Zeiss microscope x 40 objective, with its excite lamp on the maximum 4 setting =

120 watt 488nm. Within 10 mins all the EGFP has bleached. This shows a very

rapid rate of photobleaching.
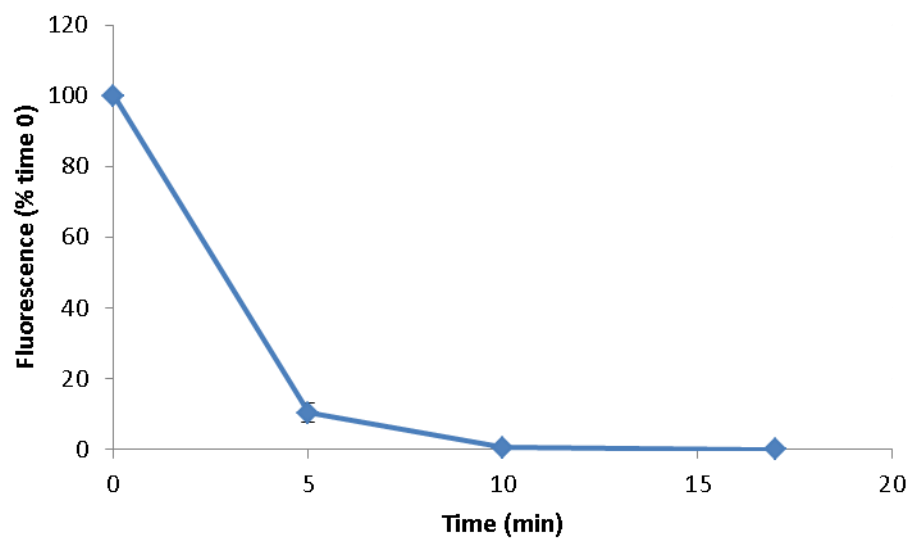
Fig.6.33. Percentage fluorescence over time for in *E.coli* recorded using the Zeiss microscope x 40 objective, with xcite lamp on the maximum 4 setting. Within 10 min all the EGFP has bleached.
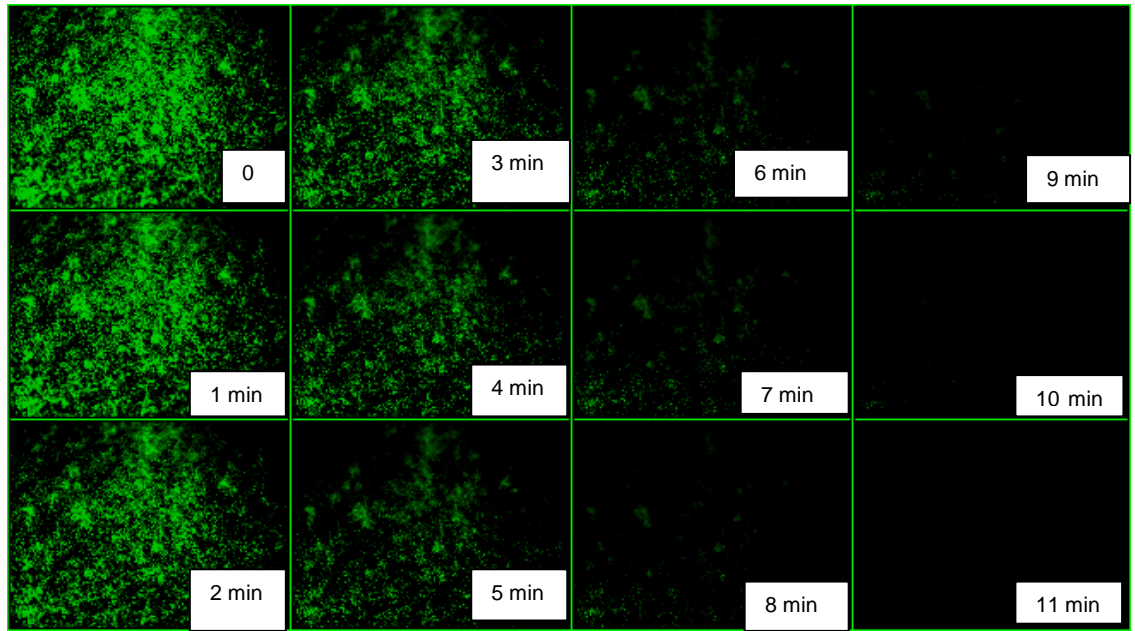
Fig.6.34. Time course recorded over 10mins for photo bleaching of EGFP in
 E. coli recorded using the Zeiss microscope x40 objective, with Xcite lamp on
the maximum 4 setting = 120 watt 488nm. Fluorescent images taken every minute,
within 10 min all the EGFP has bleached. This showed a very rapid rate of
photobleaching.

Further time course over 10 min.

Using the Zeiss microscope x40 objective, with the Xcite lamp on the maximum 4
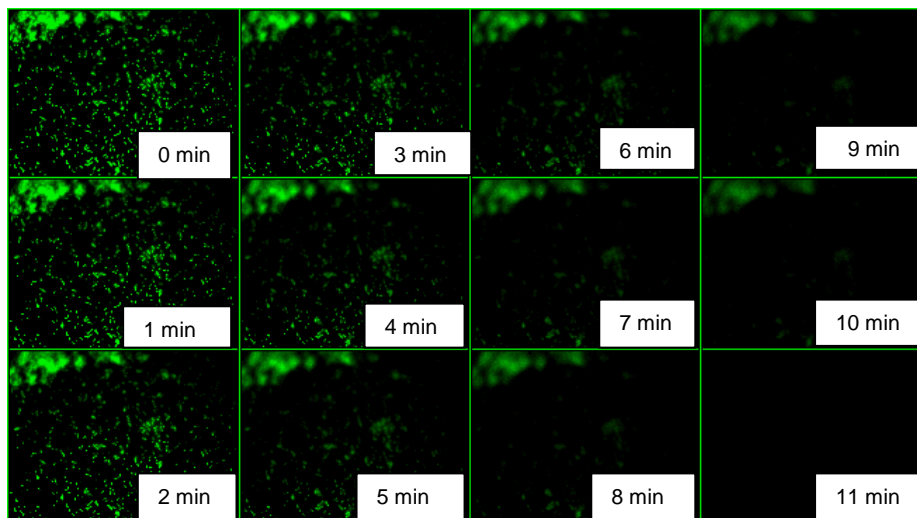
setting.

Fig.6.35. **2nd time course** recorded over 10mins for photo bleaching of EGFP in
E.coli recorded using the Zeiss microscope x 40 objective, with its Xcite lamp on
the maximum 4 setting. Fluorescent images. As in the previous experiment, within
10 min all the EGFP has bleached.
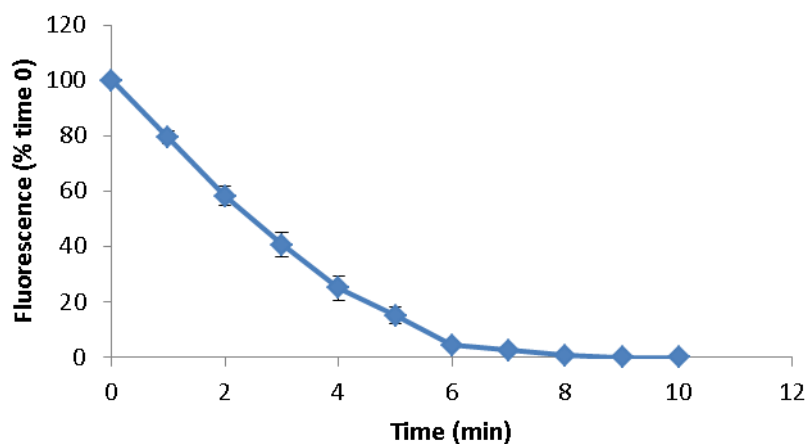
Fig.6.36. Mean fluorescence of EGFP 10 OAPS in *E .coli*.
 Using the Zeiss microscope x 40 objective, with excite lamp on the maximum 4 setting.
Mean t1/2 of photobleaching = 2.01 ± 0.8min. Range 2.84-0.79 min.
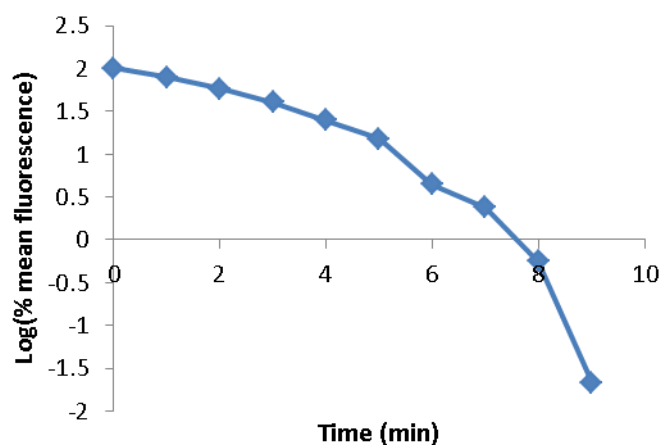
Fig.6.37. Mean log (percentage fluorescence)**.** The relationship is not linear but a bimodal distribution with two phases. These results suggest that two mechanisms may be responsible for photobleaching in EGFP.
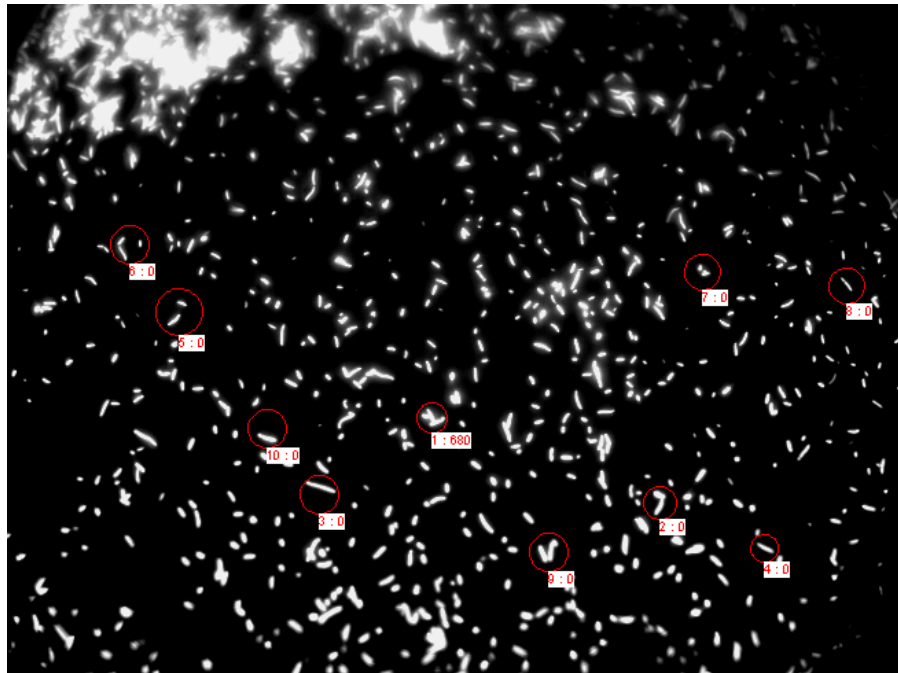
Fig.6.38. BW image showing 10 OAS for fluorescent bacteria.
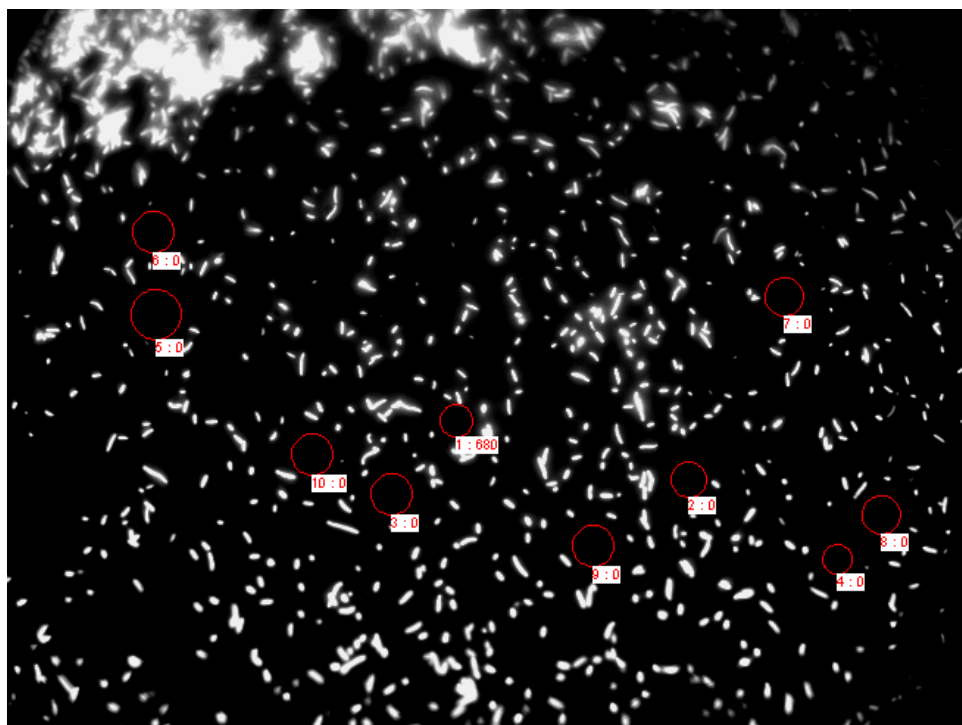
Fig.6.39. BW image showing 10 OAS for background fluorescence.
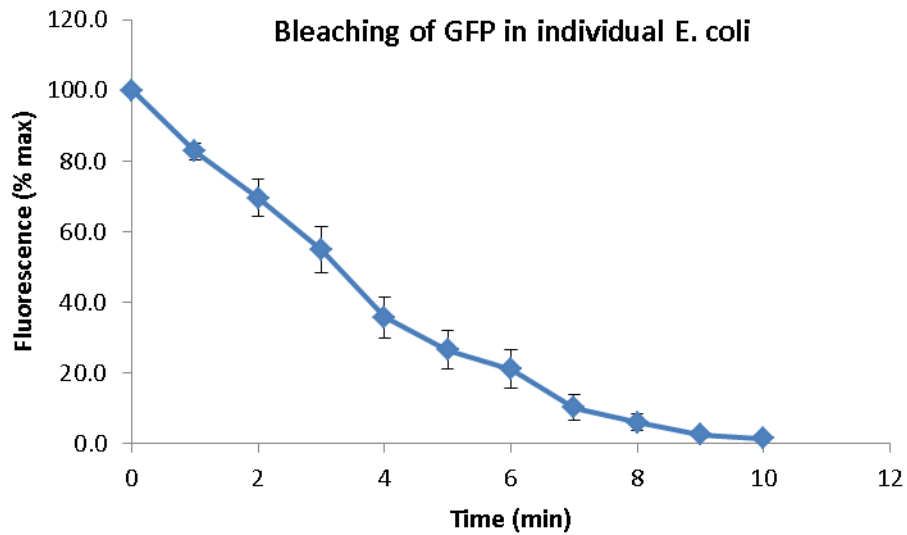
Fig.6.40. mean half-life calculated for first 6mins and then for final4mins of bleaching. Mean $t_{1/2}$ of photo bleaching = 2.56 ± 0.39minover first 6 min. Range 1.09-4.56 min. Mean $t_{1/2}$ of photo bleaching = 1.01 min over last 4 min. The difference in rates again suggests that the bleaching of EGFP may be in 2 phases.
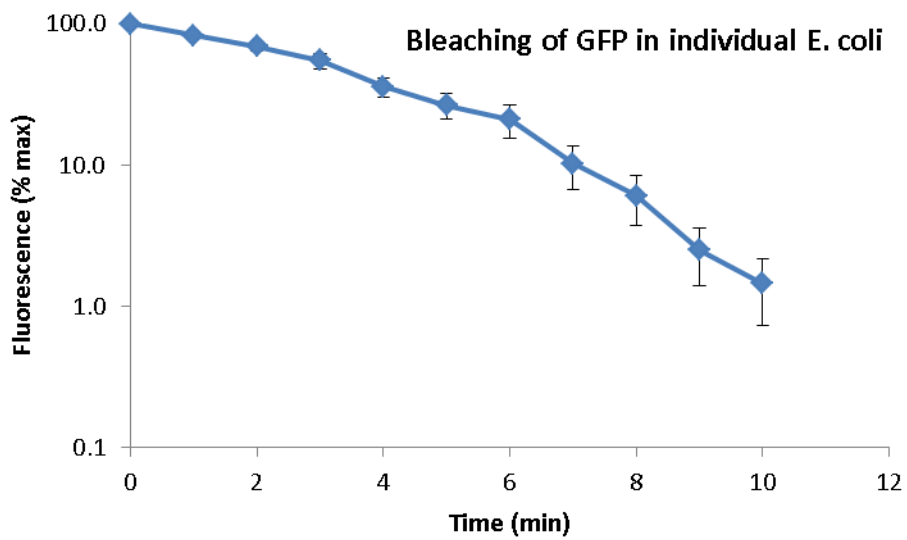
Fig.6.41. Bleaching of GFP in an individual *E .coli* cell.
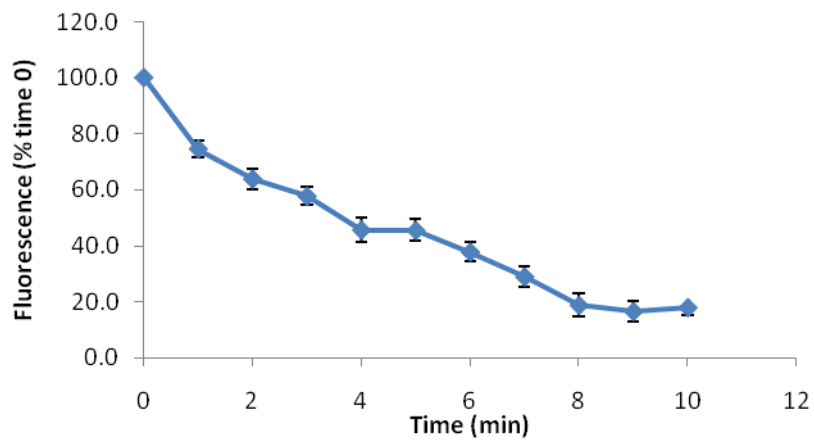
Fig.6.42. Bleaching of GFP in an individual *E. coli* cell. Xcite lamp set on 120 watt. Zeiss microscope magnification set at x 40. Mean $t_{1/2}$ of photo bleaching = 4.43 ± 0.48 min. Range 2.06 to 7.33min.

Date	Organism	GFP	Lamp	$t_{1/2}$	Range
28/9/2011	*Obelia geniculata*	Wild type	Zeiss Xcite max 4		Mean fluorescence= 81.7±5.3% Range59.4-117% at 83min
29/9/2011	*Obelia geniculata*	Wild type	Zeiss Xcite max 4	60.3± 3.1min	45.7-74.0 min
02/10/2011	*Obelia dichotoma*	Wild type	Zeiss Xcite max 4		Range at 1h 8.4-19.6%
04/10/2011	*Obelia dichotoma*	Wild type	Hund max	5.9± 0.8h	2.7-9.1h
05/10/2011	*Obelia dichotoma*	Wild type	Zeiss Xcite ½ max 2	6.6± 1.3h	3.1-15h
11/10/2011	*E. coli*	EGFP	Zeiss Xcite max 4		Fluor,10%by 5min
11/10/2011	*E.coli*	EGFP	Zeiss Xcite max 4	2.0± 0.8min	0.8-2.8min
11/10/2011	*E.coli*	EGFP	Zeiss Xcite max 4	2.6± 0.4min	1.1-4.6min
11/10/2011	*E.coli*	EGFP	Zeiss Xcite max 4	4.4± 0.5min	2.1-7.3min

Table.6.2. Summary of photobleaching of GFP. Xcite lamp set on max 4 =120watt. Hund lamp max =100watt.

6.4 Discussion and future work

The main findings of this chapter are that there was a difference in the fluorescence maxima for *Obelia geniculate* (512nm) and *Obelia dichotoma* (518nm). This indicates that the fluorescence from *Obelia geniculata* is greener

than expected. Results from dual wavelength chemiluminometry confirmed that bioluminescence produced from *Obelia dichotoma* and *Obelia geniculate* is nearly 100% green light rather than blue. This confirms that the GFP in Obelia photocytes acts as a fluor altering the wavelength of blue light produced by obelin. Photek analysis of fluorescent images showed similar RGB ratios for *Obelia* species including medusa. These showed a high level of green light, almost certainly due to GFP. A siphonophore was highly fluorescent but gave off no blue light; the cause of its fluorescence requires further investigation. Calculating RGB with Photek analysis software is on the limit of the current soft-wares capability. Repeating these experiments using FLIM would give more reliable data and could lead to a range of fluors being pinpointed. The selective advantage of green emission instead of blue emission from Ctenophores is still being sort. It does appear to be linked to the fact that Ctenophores exist at greater depth. GFP may have originally evolved as UV protection in bioluminescent Cnidarians. Photobleaching results illustrate the fact that wild type GFP in *Obelia* bleaches far slower than genetically engineered GFP in EGFP.

The results of this chapter show that GFP in *Obelia geniculata* and *Obelia dichotoma* photobleached much slower than genetically engineered GFP expressed in *E. coli*. This is illustrated in Table 6.2.and 6.3

Species	Lamp	$t_{1/2}$
GFP in *Obelia geniculata*	Excite	63.0 ± 3.1mins
EGFP	Excite	2.0 1±0.8mins

Table.6.3. $t_{1/2}$ for photobleaching of wild type GFP is much longer than that for GFP in EGFP.

This illustrates how $t_{1/2}$ for photo bleaching of GFP in *Obelia geniculata* is much longer than that for GFP in EGFP. The results also suggested that the photobleaching of GFP in *E. coli* may occur in two phases. The half-life for GFP bleaching in the first 6 minutes was longer than the half-life for the final 4mins, 2.56 ±0.39 min compared to 1.01 mins.

Trial experiments in *Obelia dichotoma* suggested that the rate of photobleaching may vary, depending on the position of photocytes. More experiments need to be carried out to investigate this.

Half-lives of photobleaching of GFP could vary in different species of *Obelia*. In *Obelia geniculata* $t_{1/2}$ was 63.0 ± 3.1mins whereas in *Obelia dichotoma* $t_{1/2}$ was 6.6 ±1.3h. However a lower powered lamp was used to measure $t_{1/2}$ in *Obelia dichotoma* so no real conclusion can be drawn until the experiment is repeated using identical lamp strengths.

There was a difference in the rate of photobleaching when different lamps were used to excite the fluorescence of the GFP. Under the Hund 100w Hg lamp *Obelia dichotoma* $t_{1/2}$ was 5.9 ± 0.8h, whereas *Obelia dichotoma* under the excite lamp 60watt gave $t_{1/2}$ at 6.6 ± 1.3h. This is probably due to the 100w lamp of the Hund microscope being slightly stronger than that of the Xcite set on half power. The

major aim of quantifying photobleaching in *wild type* GFP in *Obelia* and in EGFP was achieved.

The Photobleaching mechanism is not entirely clear in some cases it needs singlet oxygen to oxidise the fluor, whereas fluorescein is known to photobleach without molecular oxygen.

The question is does photobleaching of GFP require oxygen and what is the protection in *Obelia*? The most likely mechanisms are, either it forms a dimer to protect itself from oxidation, or it has an oxygen scavenger i.e. coelenterazine.

The mechanism is likely to involve coelenterazine which is known to be an antioxidant (Arrault et al. 2003, de Wergifosse et al. 2004, Devillers et al. 2001, Saleh and Plieth, 2010). Rees et al have described coelenterazine as being the marine equivalent of vitamin C as it has a high affinity for superoxide anion (Rees et al. 1998). Coelenterazine's antioxidant properties may partially explain why it has evolved as the most ubiquitous luciferin in marine bioluminescence.

"Bioluminescence is not essential for the survival or reproduction of luminous species". A K Campbell has predicted that the selective advantage of marine fluors could be linked to temperature control. "The selective advantage of fluorescence, including GFP and colours in corals and anemones, in many coelenterates is likely to be related to temperature. This is because fluors absorb light energy and reemit it without allowing the light energy to be converted to heat" (Campbell, 2012). Or it could be that GFP gives protection from damaging UV light. This theory is supported by the fact that GFP has been found in the snakelocks anemone, which is often found in shallow rock pools. Future work could focus on investigating whether GFP photobleaches at a different rate, depending on the position of the

photocyte it is in. More experiments could be carried out to ascertain if the rate of photobleaching is different in the 3 different species.

Chapter seven

Evolution of bioluminescence

7.1 Introduction

Eight marine phyla contain examples of bioluminescence which utilise coelenterazine as the luciferin (several examples of these have been discussed in chapter one).

It can therefore be stated that "The most common chemistry responsible for bioluminescence in the sea is coelenterazine" (Campbell & Herring 1990; Thomson et al. 1997; Campbell, 2003). A vital question which remains to be answered is how coelenterazine based bioluminescence evolved.

Darwin recognised in his origin of the species, that the widespread occurrence of bioluminescence could not be easily explained by natural selection alone.

" The luminous organs which occur in a few insects, belonging to widely different families, and which are situated in different parts of the body, offer under our present state of ignorance, difficulty almost parallel with that of electric organs" (Darwin, 1868). E. N. Harvey working in the 1950's highlighted that the random occurrence of bioluminescence in some phyla but not others, made the evolutionary pathway for bioluminescent systems difficult to predict. "It is as if the main groups of organisms are listed on a blackboard, and a handful of sand is thrown. The luminous species are where the sand sticks" (Harvey, 1952; Campbell, 2003). Scientists today are still struggling to see any pattern in the occurrence of bioluminescence. "The occurrence and known chemistry of bioluminescence exhibits no obvious biological pattern in different groups of organisms" (Herring, 1978).

J. Woodland Hastings has predicted that bioluminescence must have evolved on 30 separate occasions and occurred after the evolution of vision (Hastings, 1983; McCapra, 1990). He has also hypothesised that luciferases originally evolved due to their ability to act as" oxygenase enzymes" for vital reactions (Hastings, 1983; Herring, 1987).

More recently Campbell has formulated the solvent cage hypothesis which postulates that;

"Bioluminescent proteins may have initially evolved as a solvent cage consisting of a few key amino acids" (Vassel et al. 2012). In a classic luciferin-luciferase reaction the luciferase binds to the luciferin and will determine the colour of the light emitted.

"In fact all the luciferase does is create a solvent cage around the luciferin allowing one and only one oxygen atom in, and keeping water out (both oxygen & water quench electronically excited states" (Campbell, 2003b; Campbell, 1994; Watkins et al. 1993).

The aim of this chapter was to utilise bioinformatics to ascertain the % sequence similarities between different marine luciferases, photoproteins (containing coelenterazine), and key calcium binding proteins such as calmodulin. This would test the hypothesis made by Hastings "that bioluminescence must have evolved on at least 30 separate occasions."

A subsequent aim was to investigate the "solvent-cage hypothesis" by determining if a range of proteins including albumin would act as a catalyst for the chemiluminescence of the marine luciferin coelenterazine. The results of this

investigation would give further information on which amino acids need to be at the "active site" of a luciferase in order for it to catalyse the chemiluminescence of coelenterazine.

The strategies were;

- To utilise bioinformatics to ascertain the % sequence similarities between different marine luciferases, photoproteins (containing coelenterazine), and key proteins such as calmodulin.
- To conduct experiments using the chemiluminometer to ascertain if albumin or other common proteins would catalyse the chemiluminescence of coelenterazine.

Some of the results of this chapter have been published in Vassel et al 2012.

7.2 Materials and Methods

Materials

Coelenterazine was a kind gift from Bruce Bryan (Prolume Inc). All other reagents were Analar from Sigma.

Measurement of chemiluminescence

Chemiluminescence was measured using the chemiluminometer (details already given in chapter 2). Protein solutions were made up in 50 mM Hepes buffer pH 7.4. The volume of each experiment was 100 µl. The mean of 6x 10s counts were calculated for each run.

Sequence similarities

Sequence similarities were obtained using BLAST and CLUSTAL on sequences obtained from GenBank.

7.3 Results

The photoproteins from four bioluminescent Cnidarians (aequorin, mitrocomin, obelin and clytin) have now been cloned (Charbonneau et al. 1985; Fagan et al. 1993; Inouye & Tsuji, 1993; Illarionov et al. 1995); therefore amino acid sequences for these four proteins can be compared, using bioinformatics. An integral part of all these light producing proteins is coelenterazine, and they all consist of 195 to 198 amino acids. The results in Table 7.1 illustrate that mitrocomin, obelin and clytin all show a high percentage of sequence similarity with aequorin. This is not surprising as they are in the same class (hydrozoan) and the same phylum (Cnidarian). There is a far lower percentage similarity between aequorin and berovin from the comb jelly *Beroe ovata,* which is probably due to the fact that *Beroe ovata* is from a different phylum i.e. Ctenophore rather than Cnidarian. All the photoproteins contain coelenterazine and all are triggered by binding calcium cations, but their percentage similarity with aequorin ranges from 22 to 69%.

This supports the hypothesis of Hastings that bioluminescence "must have evolved on 30 separate occasions" (Hastings, 1983: McCapra, 1990). Table.7.1. illustrates the particularly low sequence similarity between *Renilla* luciferase and aequorin (15%) even though they occur in the same phylum and both use coelenterazine to produce bioluminescence. Also the percentage

similarity of aequorin to the luciferase of *Gaussia princeps* an arthropod was extremely low (5%).

Table 7.2 shows that the sequence similarities are even lower when *Renilla* luciferase is compared to a range of arthropoda luciferases, these range from only 2 to 4%. Table 7.3 illustrates that sequence similarities remain high within the same class and phylum e.g. *Gaussia princeps* luciferase is 68% similar to *Metridae longa* luciferase. These are both species of copepods which are known to contain coelenterazine.

Calmodulin is a human protein which binds calcium ions and is involved in calcium signalling. It has been suggested that calmodulin may be the protein from which photoproteins such as obelin and clytin evolved. However when the sequence similarities are compared there is only a relatively low commonality of 24% between aequorin and calmodulin. This level of similarity can be explained by the fact that both proteins bind calcium using 3 EF hand sites. Calmodulin does not have the essential sequence of amino acids needed for the solvent cage.

Protein	Organism	Protein ID	Common name	Phylum	Class	Amino acids	% similarity with aequorin
Photoproteins							
Aequorin	*Aequorea aequorea*	AAA27719	Jelly fish	Cnidaria	Hydrozoa	196	100
Mitrocomin	*Mitrocoma cellularia*	AAA29298	Jelly fish	Cnidaria	Hydrozoa	198	69
Obelin	*Obelia geniculata*	AAL86372	Hydroid	Cnidaria	Hydrozoa	195	64
Clytin	*Clytia hemisphericum*	AAA28293	Jelly fish	Cnidaria	Hydrozoa	198	62
Berovin	*Beroe ovata*		Comb jelly	Ctenophora	Nuda	208	22
Luciferase							
Renilla	*Renilla reniformis*	AAA29804	Sea pansy	Cnidaria	Anthozoa	311	15
Oplophorus (19kda)	*Oplophorus gracilorostris*	BAB13776	Shrimp	Arthropoda	Decapoda	196	5
Oplophorus (35kda)	*Oplophorus gracilorostris*	BAB13775	Shrimp	Arthropoda	Decapoda	359	11
Gaussia	*Gaussia princeps*	AAG54095	Copepod	Arthropoda	Copepoda	185	5
Metridia	*Metridia longa*	AAR17541	Copepod	Arthropoda	Copepoda	219	2
Other proteins							
Calmodulin	*Homo sapiens*	BAA08302	Human	Chordata	Mammalia	149	24
Albumin	*Homo sapiens*	NP_000468	Human	Chordata	Mammalia	609	7
GFP	*Aequorea aequorea*	CAA58789	Jelly fish	Cnidaria	Hydrozoa	238	7
GFP	*Renilla reniformis*	AAK54757	Sea pansy	Cnidaria	Anthozoa	233	5

Table 7.1 Sequence similarities of aequorin when compared with photoproteins containing coelenterazine, luciferase and other relevant proteins (Vassel et al. 2012).

234

Luciferase	Organism	Protein ID	Common name	Phylum	Class	Amino acids	% similarity with *Renilla* luciferase
Renilla	*Renilla reniformis*	AAA29804	Sea pansy	Cnidaria	Anthozoa	311	100
Oplophorus (19kda)	*Oplophorus gracilorostris*	BAB13776	Shrimp	Arthropoda	Decapoda	196	2
Oplophorus (35kda)	*Oplophorus gracilorostris*	BAB13775	Shrimp	Arthropoda	Decapoda	359	4
Gaussia	*Gaussia princeps*	AAG54095	Copepod	Arthropoda	Copepoda	185	4
Metridia	*Metridia longa*	AAR17541	Copepod	Arthropoda	Copepoda	219	3

Table 7.2. Sequence similarity between *Renilla* luciferase and luciferases from Arthropoda (Vassel et al. 2012).

Luciferase	Organism	Protein ID	Common name	Phylum	Class	Amino acids	% similarity with *Gaussia* luciferase
Gaussia	*Gaussia princeps*	AAG54095	Copepod	Arthropoda	Copepoda	185	100
Metridia	*Metridia longa*	AAR17541	Copepod	Arthropoda	Copepoda	219	68

Table 7.3. Sequence similarity between two copepod luciferases, *Gaussia* and *Metridia* (Vassel et al. 2012).

Results protein experiments
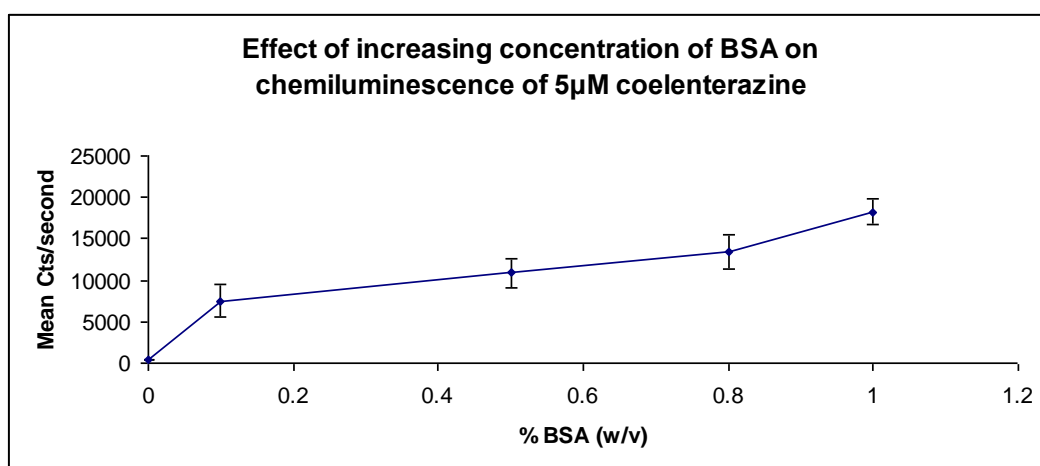
Fig.7.1. Effect of increasing concentration of BSA. Effect of increasing concentration of BSA on chemiluminescence of 5μM coelenterazine. Chemiluminescence was recorded as 6x10s counts and the mean was taken. Results represent the mean ±SEM of 3 experiments.
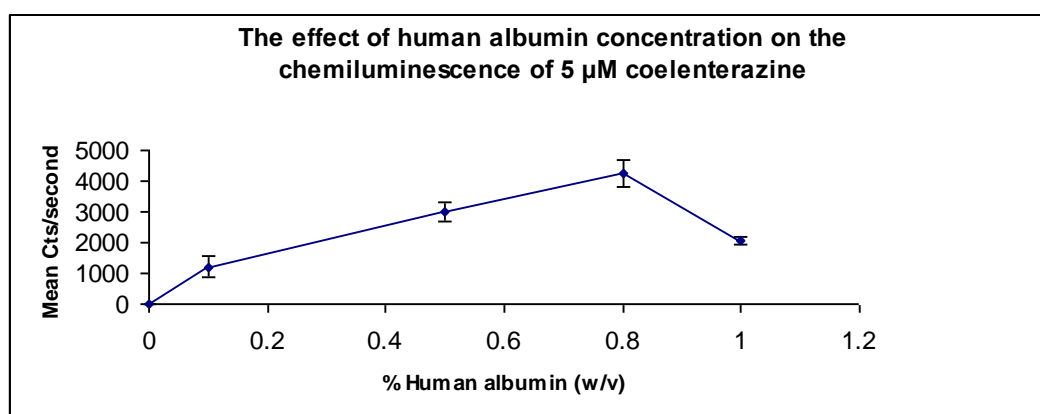
Fig.7.2. Effect of increasing concentration of human albumin on chemiluminescence of 5 μM coelenterazine. Chemiluminescence was recorded as 6x10s counts and the mean was taken. Results represent the mean ±SEM of 3 experiments.
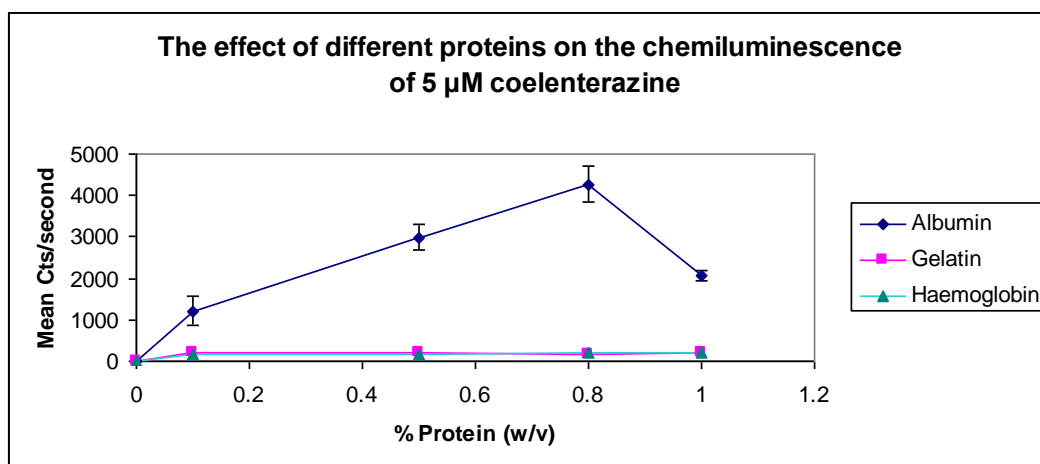
Fig.7.3. The effect of different proteins on the chemiluminescence of 5 μM coelenterazine. Chemiluminescence was recorded as 6x10s counts and the mean was taken. Results represent the mean±SEM of 3 experiments.
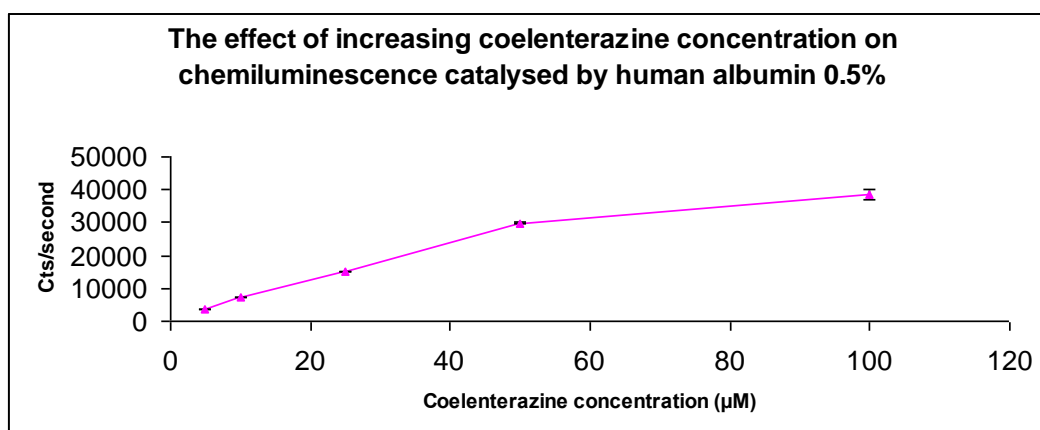
Fig.7.4. The effect of increasing coelenterazine concentration. The effect of increasing coelenterazine concentration on the chemiluminescence catalysed by 0.5% human albumin. Chemiluminescence was recorded as 6x10s counts and the mean was taken. Results represent the mean±SEM of three experiments.

The results of the protein experiments confirmed that in a polar solvent coelenterazine will produce a low level of chemiluminescence, which increases with increasing concentrations of this luciferin. Human serum albumin will slightly increase this effect, but bovine serum albumin markedly increases

coelenterazine chemiluminescence (Fig 7.1). Fig 7.3 illustrates that gelatin and

human haemoglobin do not catalyse coelenterazine chemiluminescence.
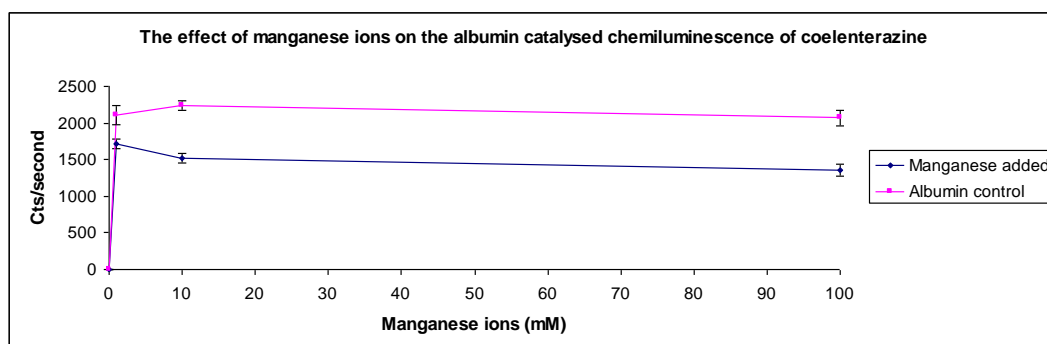
Fig.7.5. The effect of manganese ions. The effect of manganese ions on the albumin catalysed chemiluminescence of coelenterazine. Chemiluminescence was recorded as 6x10s counts and the mean was taken. Results represent the mean ±SEM of 3 experiments.

The presence of manganese ions lowered the intensity of albumin catalysed

chemiluminescence of coelenterazine. This was to be expected as Manganese ions

will bind to human albumin or bovine serum albumin. The effect of cobalt and

zinc ions was also investigated but is not presented.

7.4 Discussion

It has still not been established as to why and how bioluminescence evolved. Considering Charles Darwin's theory of evolution it can be predicted that the DNA coding for bioluminescent proteins must confer some survival advantage, otherwise it would not have been conserved in so many species.

The fossil records contain examples of bioluminescent organisms that are still living today. This demonstrates that bioluminescence existed as far back as 80 million and even 200 million years ago. Examples of fossilised bioluminescent species include fireflies preserved in amber resin which are thought to have lived 80 million years ago (Harvey, 1952).The Natural History Museum in London has a fossilised specimen of a lantern fish which lived in the oceans approximately 100 million years ago. There are also examples of fossilised marine specimens which bear a close resemblance to *Amphipholus squamata* (Fig. 7.1). Vassel et al state that "Fossil brittle stars appear, identical to the contemporary luminous brittle star, *Amphipholus squamata*, some 200 million years old" (Vassel et al. 2012).

Fig.7.6. A fossilised brittle star (Vassel et al. 2012).

239

J. Woodland Hastings has predicted that bioluminescence must have evolved on 30 separate occasions and occurred after the evolution of vision (Hastings, 1983: McCapra, 1990). He has also hypothesised that luciferases originally evolved due to their ability to act as oxygenase enzymes for vital reactions (Hastings, 1983: Herring, 1987). When exactly different bioluminescent proteins evolved was investigated here by comparing the amino acid sequences of their DNA (see also Vassel et al. 2012). If Hastings hypothesis is true only low levels of amino acid sequence similarities between bioluminescent proteins were predicted. When the sequences of luciferases from different phyla are compared sequence similarities are very low. This data supports Hastings theory.

The results of the protein experiments confirmed that in a polar solvent coelenterazine will produce a low level of chemiluminescence which increases with increasing concentrations of this luciferin. Human serum albumin will slightly increase this effect, but bovine serum albumin markedly increases coelenterazine chemiluminescence. Fig 7.3 illustrates that gelatin and human haemoglobin do not catalyse coelenterazine chemiluminescence.

Results confirm that albumin should not be used instead of gelatin in the coelenterazine assay. Albumin in human and animal blood will interact if luciferases are used in *vivo* (Vassel et al. 2012).

The effect of zinc and cobalt ions was also investigated, but is not presented. Repeat experiments on the effects of a range of metal ions on albumin catalysed coelenterazine chemiluminescence, using lower concentrations of metal ions i.e. 0.01 to 10 mM now needs to be carried out.

3D molecular modelling performed by Vassel et al confirms that albumin contains Sudlow site I which will bind coelenterazine (refer to Fig 7.7). The site has important amino acids for the binding of colenterazine i.e. Tyr 150, Lys 195, Arg 222, His 242, and Arg 257 (Vassel et al. 2012). "Similar basic amino acids have been found in the coelenterazine binding site of aequorin and obelin" (Vassel et al 2012; Vysotski et al. 2004). The results show that human and bovine serum albumins have mono-oxygenase activity, and therefore support the solvent cage hypothesis. Watkins and Campbell have already demonstrated that "the c-terminal proline in aequorin is essential for its maximum activity" (Watkins & Campbell, 1993). The c-terminal proline is an essential part of aequorin's structure. It is the 189th amino acid in aequorin's structure. Proline 189 confers stability on the structure of the photoprotein. Other photoproteins including obelin also have this proline. The proline in obelin is not in same position, and may not have the same function. When compared to other calcium binding proteins obelin and aequorin have relatively high numbers of tryptophan, cysteine and histidine residues in their structure. This suggests that these amino acids are vital to the light producing function of the proteins. These amino acids are probably in a functional domain for light production. Further work is needed, but all these results support the prediction that bioluminescent proteins may have originally evolved as primitive enzymes (Vassel et al. 2012, Campbell, 2012).
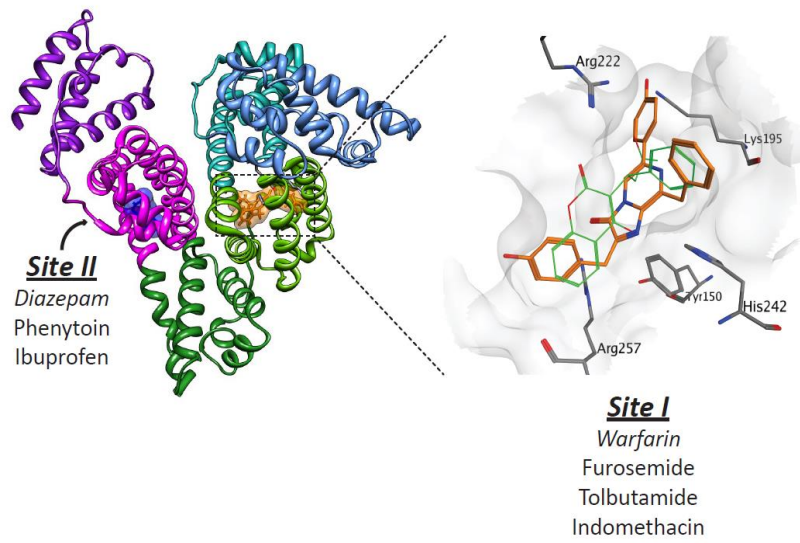
Fig 7.7. 3D molecular model of human albumin. (a) Illustrates the Sudlow site I where coelenterazine is thought to bind to albumin. (b) Sudlow site II. Coelenterazine is represented in orange and key amino acids thought to be involved in its binding are highlighted, i.e. tyr 150, lys 195, arg 222, his 242 and arg 257.Warfarin is represented in green as a comparison (Vassel et al. 2012)

Chapter eight

Bioluminescence in Education

8.1 Introduction

Having taught science in further education for over 22 years, the difficulties of interesting young people in scientific topics are very apparent. Having worked with Prof Anthony Campbell to set up the Pembrokeshire Darwin Science festival since1999, it was soon obvious that bioluminescence is a topic which never fails to interest and engage young people from all walks of life. The key question was whether bioluminescence could be used to teach a wide range of scientific skills and principles. Can bioluminescence be an "educational tool?"

8.1.1 Dr Darwin's curiosity shop

Fig.8.1. Dr Darwin's curiosity shop. Presenting the hands on exhibit at the Edinburgh science festival, where over 1000 members of the public visited the exhibit.

The "hands on" exhibit was named after Charles Darwin's grandfather Erasmus Darwin. Members of the public and school pupils entered a black tent in which they participated in demonstrations and hands-on exhibits .This allow different aspects of bioluminescence to be introduced and explored. Another key theme covered in the presentation is the difference between fluorescence and bioluminescence. The topic of bioluminescence was covered by posing a series of questions:-

How is a flash or glow produced?

This compared incandescence with chemiluminescence. The heat associated with a candle flame was measured using a thermocouple and other terrestrial incandescent light sources discussed. This was followed by the chemical basis of chemiluminescent reactions. A demonstration of the three essential components, producing the "blue glow" (refer to methods 8.2).

By adding 1 ml 100 mM fluorescein to a second demonstration the role of GFP in altering the wavelength of bioluminescent light was discussed. Live specimens of *Obelia geniculata* or *Obelia longissima* were viewed under the fluorescent microscope as an example of marine bioluminescent organisms which contain GFP.

2. What is bioluminescence?

Different examples of bioluminescent organisms were introduced to the audience, using images, live organisms e.g. glow-worms, brittle stars and dinoflagellates, but also preserved specimens e.g. sea-pens and dragon fish. Interestingly the preserved

specimens created as much interest as the living ones. This introduced concepts of naming organisms and classification.

3. Why does it occur?

This section covered the different applications by living organisms including the angler fish's lure, and dragon fish's use of red light (torch demo refer to methods)

4. Where does it occur?

Terrestrial and marine examples were given to the audience. When presenting to Pembrokeshire schools we stressed the specific examples of bioluminescent organisms in Pembrokeshire e.g. *Amphipholas squamata.*

5. When does it happen?

The circadian rhythm in bioluminescent dinoflagellates was discussed, together with the piddock's *Pholas dactylus* links with the lunar cycle. It is difficult to trigger *Pholas's* bioluminescence during a full moon but it has yet to be confirmed if this is due to a circadian rhythm (Dunstan et al.2000).

6. So what can we do with it?

Applications discussed particularly medical, including measurements of calcium ions.

A version of the curiosity shop linked to the concept of rainbow proteins was invited to appear at the Royal society's summer exhibition. We were part of the team that demonstrated the exhibit, which was voted the top exhibit for that year.

The demonstrations in the tent were then developed into "the oceans road show" which also covered other key topics, including food chains and camouflage. These were presented to larger junior school audiences and were funded by Education and learning Wales (ELWA) an assembly sponsored public body.

Fig.8.2. Demonstrating chemiluminescence on the oceans' road show. Image must not to be reproduced without the permission of the Darwin centre for Biology and Medicine.

From March 1999 the educational program for the Pembrokeshire Darwin Science Festival began to evolve. The festival was run by the Darwin Centre for Biology and Medicine. Having accepted the role of education officer a range of scientific events were planned with the emphasis on bioluminescence and cutting edge science.

Bioluminescence was used to excite curiosity in the festival participants and inspire them to want to discover more about the science behind the phenomenon. Hence our motto "curiosity inspires discovery reveals". In our presentations and

workshops we aimed to stress the key scientific skills which Charles Darwin used so efficiently in producing his impressive body of scientific work. In particular we focused on his keen observation and meticulous recording, as well as his love of natural history and geology. A series of science events were held, which included 4 bioluminescent based activities.

- A presentation on bioluminescence in the deep sea

- Seashore search for bioluminescent marine creatures

- A DNA workshop with hands on activities stressing the DNA sequences in luminous jelly fish and glow-worms.

- The Great millennium glow-worm hunt piloted in 1999 and then Wales wide in 2000 linked with BBC radio Wales. Glow-worm hunt kits were sent out to groups across Wales. The Glow-worm gossip chat room was used for reports on what groups had seen. Organised hunts were held at Fresh water, Tenby, Penarth and Snowdonia. Darwin centre staff gave live broadcasts from the various hunts. What's a glow-worm's favourite colour? Was an experiment linked to the glow-worm hunts. Glow-work hunts have remained a popular feature of our annual festival.

In 2000 the festival secured a millennium commission grant to hold a larger range of events to celebrate the millennium. This included a series of scientific lectures, competitions and field trips.The festival has used bioluminescence to stimulate interest in natural history and the marine environment. The activities have stressed the importance of developing skills in scientific method such as recording, observing and analysing. Many participants have gone on to carry out their own

scientific investigations and achieved Crest science awards at our annual celebration of science "science aglow." Crest science awards are centered on student projects covering the subject areas of science, technology, engineering and mathematics (STEM). The awards are nationally recognised and awarded by the British science association at either a bronze, silver or gold level. To receive an award a student must make correct use of scientific method, producing a report which includes a hypothesis, experimental design, methods, results and conclusions.

By carrying out investigative projects in bioluminescence students learned a variety of key scientific principles, experimental method and gain an insight into the latest developments in cutting edge science. Since 2000 1228 Crest awards have been gained by individuals of all ages through their work with the festival. They also improved their skills in taxonomy and microscopy. Some took an interest in the Photek imaging system and fluorescent microscopy; such systems involving ICCD are now on the extended national diploma syllabus. Microscopy in general is on the A level biology syllabus. The project is also an exciting vehicle for them to gain their key skill qualifications which are increasingly important in post 16 education e.g. working with others, presentation skills, numeracy, communication and ICT. Many junior school students have also carried out projects helping them to cover the key stage areas of interdependence of organisms, life cycles and food chains, but also to understand how to use the binomial system of naming species to classify living organisms. They are encouraged to construct experiments as" fair tests "improving their abilities to plan experiments, develop methods and reflect on results. Students also tell us that

being able to design the project themselves is very enjoyable as they are not often given any choice in what they study. The projects also contribute towards the movement for "education outdoors" allowing students to develop fieldwork skills from an early age. Most projects had scientific mentors which included myself. Many other mentors were either local experts e.g. Dr Robin Crump or scientists from Cardiff university e.g. Prof Anthony Campbell.

Dinoflagellate project

Crest project conducted with national diploma in health studies students at Pembrokeshire College. These students had all applied to study nursing or physiotherapy at University. They had taken part in a glow-worm hunt at Freshwater East (Pembs) and were fascinated to also observe the bioluminescence on the beach there, as waves hit the beach in relative darkness. They decided to carry out a project on bioluminescent dinoflagellates which included research on the general applications of bioluminescence in medicine. Students also researched how dinoflagellates use bioluminescence as a burglar alarm to deter predators. Following my introductory lecture on bioluminescence and talking to Prof Anthony Campbell the students decided to set up experiments on the effect of circadian rhythm on dinoflagellate bioluminescence. Dinoflagellates have a circadian rhythm and only produce bioluminescence at night. The species they chose to investigate was *Pyrocystis lunula*. The results showed that by placing dinoflagellates on a reverse light cycle you could also reverse the circadian rhythm of their bioluminescence.

8.2 Methods and materials

Culturing bioluminescent dinoflagellates.

Initially Temperature of lab had to be monitored using a datalogger as dinoflagellate bioluminescence is effected if they are kept below 15^0C.This showed that the temperature only varied between 19.5 and 21^0C over several days. The datalogger used was obtained from Phillip Harries.

Dinoflagellate *Pyrocystis lunula*

50 ml starter cultures were obtained from Joe Andrade's centre for integrated science education at Utah University 2480 MEB Salt Lake City UT84112.Unfortunately the cultures are no longer available from this source but there are alternative suppliers. Cultures of the dinoflagellates were set up in plastic milk containers which had been carefully washed out. The method of culturing them was based on information from Dr Joseph Andrade. Artificial seawater was produced by dissolving 31.8g of marine salt in to one litre of tap water. Batch A (6 X500ml containers) were kept on the window sill of the lab and were on a normal light cycle.

Reversing the light cycle of *Pyrocystis lunula*

 Batch B (6 X500 ml containers) were kept in a light box which was constructed by Peter Gleed the college technician. The main components of this were a fluorescent tube and timer switch. The light box ensured that during the day the dinoflagellates received no light whereas they were fully illuminated at night.

Observing and counting dinoflagellates

To view the dinoflagellates under the microscope they needed to be slowed down. Several drops of 0.36 M $MgSO_4$ were placed over dinoflagellates on a microscope slide. This slowed them down sufficiently so that they could be viewed or counted.

Demonstrations of chemiluminescence

<u>The blue bioluminescence flash</u>

In 200 ml beaker /flask

50 ml Buffer L

1ml 10 mM luminol

0.5ml 100mM cupric ammoniacal sulphate (1 to 2mls for a faster short lived reaction).

Darken room or tent.

Add 20 to 30 mls 0.5% H_2O_2 to produce luminescence.

<u>The green bioluminescence flash</u>

To the previous blue bioluminescence cocktail, add 1ml 100 mM fluorescein .

Add 30 mls 0.5% H_2O_2 to produce green light.

100 mM cupric ammoniacal sulphate

This is 100mM copper sulphate and 100 mM ammonium carbonate.

Use 50 mls 1M copper sulphate and 50 mls 1 M NaOH then add solid ammonium carbonate until the ppt dissolves.

Buffer L

5:1 1 M sodium carbonate to 1 M sodium hydrogen carbonate.

Demonstrating the existence of GFP in living organisms

Fresh specimens of either *Obelia geniculata* or *Obelia longissima* were viewed under the Hund fluorescent microscope to show the presence of GFP in photocytes.

Demonstrations in the black tent

Incandescence "hot light" compared with "cold light"

A thermocouple was used to measure the heat produced by a candle and different "hot" sources of light on earth discussed.

"Burning without fire"

The 3 essential chemical components of a chemiluminescent reaction were demonstrated and related to the seminal work of Dubois in discovering luciferin and luciferase.

Luminous dinoflagellates

Colonise cultured in plastic bottles, can be shaken in the darkness of the tent to demonstrate the blue luminescence they produce.

Why are shrimp red?

Different coloured torches, the first red, the second blue are shone on a picture of a red shrimp to demonstrate how the colour of an organism can act as camouflage. The use of the dragon fishes red bioluminescence as a "sniper scope" to hunt for red prey was also introduced.

Fluorescence

The difference between fluorescence and bioluminescence demonstrated using an ultra violet lamp and fluorescent minerals. When a sheet of glass is placed between the mineral and the UV lamp the fluorescence fades. This proved it needed to be kick started by energy of UV.

Triboluminescence

This was demonstrated using Glow-stones.

8.3 Results from Crest award investigation into dinoflagellates.

Growth curves for *Pyrocystis lunula*
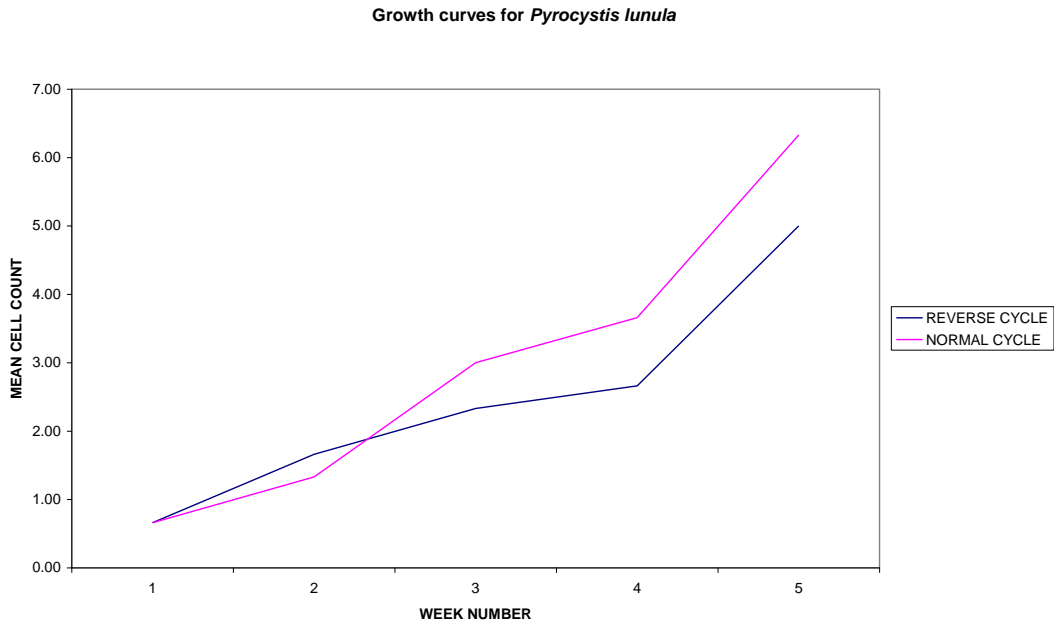
Fig.8.3. Growth curves produced by students counting dinoflagellate numbers every week using a haemocytometer, to calculate the mean cells cm^3. They measured this for both batches of dinoflagellates i.e. those on the normal light cycle and those on the reverse light cycle.
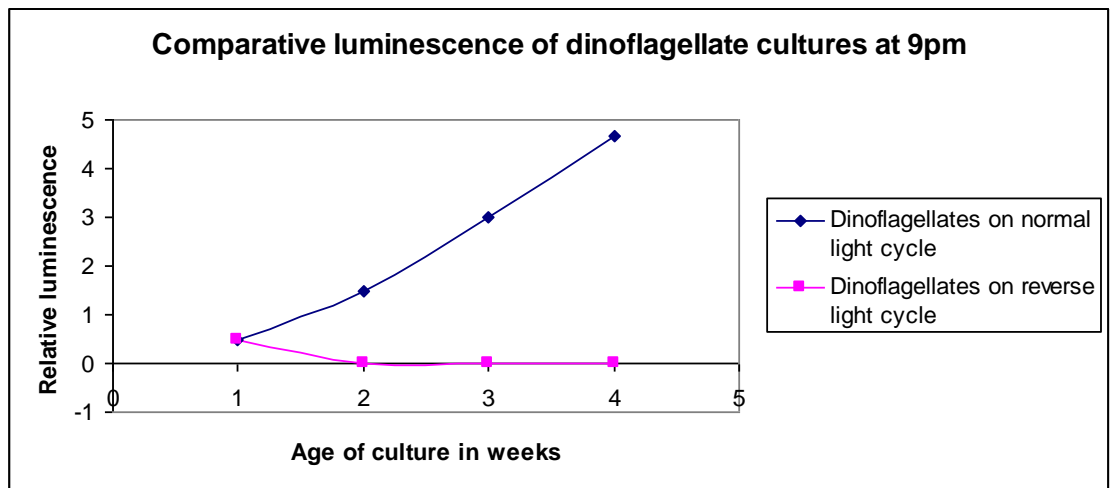
Fig 8.4 Comparative luminescence of dinoflagellate cultures recorded at 9pm weekly. Luminescence was measured on an arbitrary scale from 1 to 5 by shaking cultures in a darkroom. Six cultures were measured on each light cycle and the mean taken. By reversing the light cycle of the cultures it appears that the circadian rhythm of the dinoflagellates bioluminescence has also been reversed.
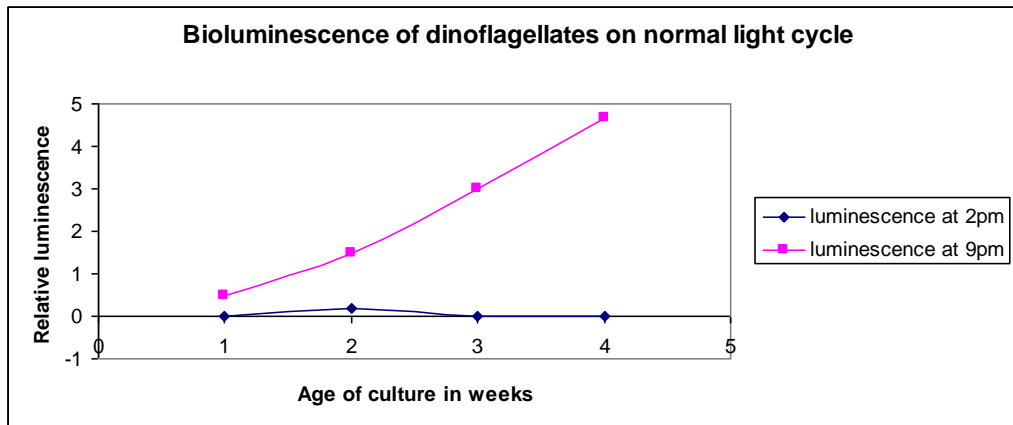
Fig 8.5 Normal light cycle. Luminescence was assessed at 2pm and then again at 9pm.
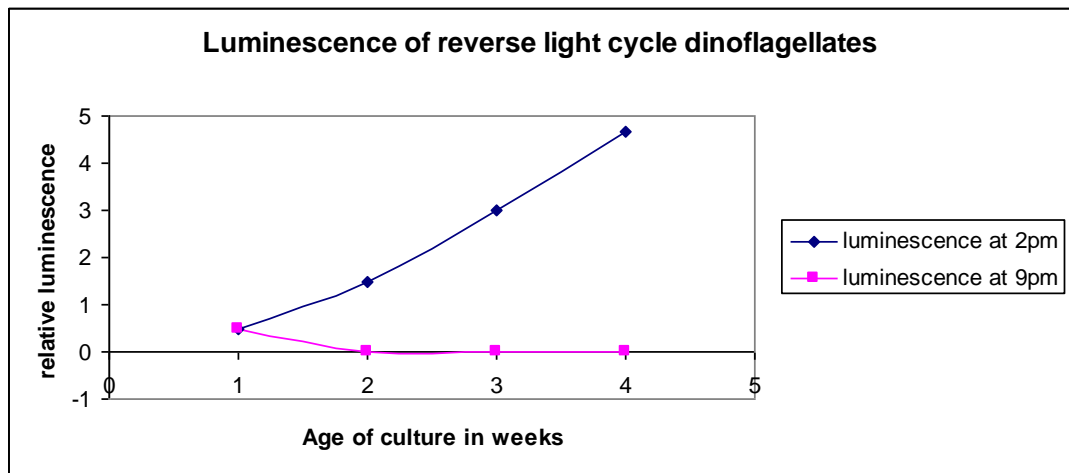
Fig 8.6 Reverse light cycle. The normal circadian rhythm has been affected. Dinoflagellates are luminesing more in the afternoon than in the evening.

Students also used the chemiluminometer to measure the bioluminescence of some of the dinoflagellate cultures.

Fig.8.7. Students receiving their silver Crest Science awards for their project on dinoflagellate bioluminescence. Image must not to be reproduced without the permission of the Darwin centre for Biology and Medicine

Fig.8.8. Students presenting their project at "Science Aglow". They explained their experiments to the judges including Howie Watkins from the BBC. Image must not to be reproduced without the permission of the Darwin centre for Biology and Medicine

257

Other bioluminescent based Crest projects included; Brittle stars e.g. *Amphipholas squamata,* and chemiluminescent clock, investigating how the reaction produces either a rapid flash or a slow glow depending on the concentration of the chemical components. Students appeared on S4C with their projects. Many of the Crest projects were produced by FE students at Pembrokeshire College, and for this and the whole concept of the festival the college was awarded a Beacon award in 2002. As part of the festival myself and Prof Anthony Campbell founded the Pembrokeshire branch of the BA.

In 2001 Crest project junior and senior winners were taken to Florence as part of European science week. The Darwin, Da Vinci, Linnaeus initiative allowed them to participate in a scientific meeting. A bioluminescent workshop was presented to teachers at the event and crest project work displayed. Students from Pembrokeshire College won a top award.

More recently a Marine research lab & seminar room set up at Milford Haven with the support of MHPA. Young adults have assisted with research into bioluminescent hydroids together with the other marine species that grow on the sampling boards and ropes. An alien species of sea squirt has been identified which probably arrived in the haven on an oil tanker. The adults have particularly developed their microscopy and taxonomy skills during the project. Some groups have investigated the link between bioluminescence and pollution. They rechecked the water quality at the sample sites measuring coliform bacteria levels as well as oxygen and nitrate levels. For many this was their first experience of field work, and they showed tremendous enthusiasm for collecting the samples. Workshops have also been held at the lab. To celebrate Darwin's anniversary, we

combined the story of Darwin's voyage on the beagle with art. Pupils drew

hydroids from the haven and were then encouraged by artists Gwen and Bob to

draw in pastels and print their favourite flora and fauna on the Galapagos

Fig.8.9. "Living light" a workshop for teachers in Florence. Image must not to be reproduced without the permission of the Darwin centre for Biology and Medicine.

Fig.8.10. The Crest project winners at European Science week in Florence 2001. Image must not to be reproduced without the permission of the Darwin centre for Biology and Medicine.

Fig.8.11. Building an ocean food chain on the road show. Image must not to be reproduced without the permission of the Darwin centre for Biology and Medicine

Fig.8.12. A Question session on the oceans road show. Image must not to be reproduced without the permission of the Darwin centre for Biology and Medicine.

Fig.8.13. Demonstrating the difference between fluorescence and bioluminescence. Image must not to be reproduced without the permission of the Darwin centre for Biology and Medicine.

Marine Futures

Barclays PLC funded the development of a series of hands on workshops designed by myself looking at the threat to the marine environment from oil pollution, global warming and beach litter. Bioluminescence as an indicator of ocean ecology was also covered. A Copus grant then followed to extend this work. The workshops formed the building blocks for the Pembrokeshire extended access to science and technology project (PEASAT) which is now funded by Dragon LNG.

Marine conference

Local marine scientists attended, together with international marine scientists and students. Speakers included Dr Sue Burton explaining the Pembs marine SAC; Dr Jim Morin speaking on bioluminescence; Anthony Campbell on sabbatical Cymru and Prof Ken Wann on the marine pharmacy.

Fig.8.14. The Marine conference. Image must not to be reproduced without the permission of the Darwin centre for Biology and Medicine.

Interseed an Interreg IIIA project linked with the Institute of technology Carlow Ireland. The focus in Pembrokeshire was in interacting with over 16's and community groups whereas the focus in Carlow was on junior pupils and community groups. Six marine bioluminescent workshops were presented during the project. All were very well attended by interested adults. A research laboratory was set up. The first two workshops concentrated on investigating the range of marine life which utilises bioluminescence as well as the medical and commercial applications of bioluminescence. Participants used microscopes to identify a range of bioluminescent hydroids and medusae. The next 2 workshops gave an insight into the research on local bioluminescent hydroids in Milford Haven waterway. Activities included viewing GFP in hydroids under the fluorescent microscope. A range of scientific posters on bioluminescence were displayed and the process of producing a good scientific poster was discussed.

Fig.8.15 Inter seed bioluminescent workshop. Image must not to be reproduced without the permission of the Darwin centre for Biology and Medicine

A Workshop on GFP was given working with Irish scientists including Dr Kieran Germaine. This included transforming bacteria to make them fluorescent and a discussion on how GFP is used as a reporter gene in environmental work e.g. bioremediation of DDT.

Fig.8.16. The GFP workshop on transforming bacteria. Image must not to be reproduced without the permission of the Darwin centre for Biology and Medicine.

The final workshop was on marine DNA and bioinformatics. DNA was extracted and analysed from a variety of sources during the workshop. The internet was utilised to search for DNA sequences involved in bioluminescence.

A research project on bioluminescent hydroids in Pembrokeshire was linked with Ireland. Cross border research seminar held. Adults and student assisted with the research. Students benefited from this experience and included it in their personal statements for University. This helped them secure places at top universities to study Biology (Imperial), Biochemistry (Aber) and Oceanography (Southampton).

8.4 Discussion and future work

The Peasat project has now been developed into the Darwin experience by Marten Lewis who has gained funding for the work from Dragon LNG. He now has an assistant Sam Williams and between them they run an incredible number of PUSH activities, from the Darwin centre of biology and medicine.We are now looking to develop the marine biology laboratory in Milford Haven as a PUSH centre. Prof Anthony Campbell has made a fantastic contribution to the work of the festival and I really appreciate his input to the events. Our numerous festival helpers have also made an essential contribution. The experience has shown that bioluminescence and fluorescence can be successfully used to produce educational packages covering a range of topics.

Fig.8.17. Demonstrating the use of red bioluminescence in the ocean depths. Image must not to be reproduced without the permission of the Darwin centre for Biology and Medicine.
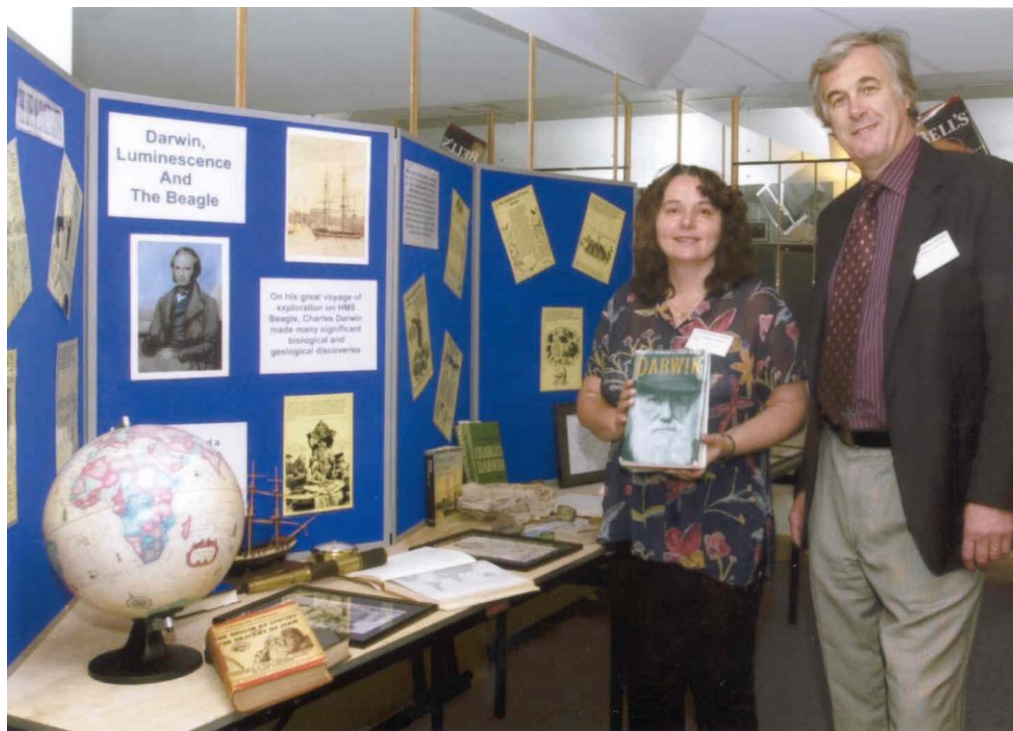
Fig 8.18. Charles Darwin lecture.

Fig.8.19. Junior art and science workshop. Image must not to be reproduced without the permission of the Darwin centre for Biology and Medicine.

CHAPTER 9

GENERAL DISCUSSION

9.1 Main findings

The major findings of this thesis were as follows; Cultures of *Obelia geniculata* fed on non luminous *Artemia* rapidly lost their bioluminescence, indicating that the hydroid requires a dietary supply of coelenterazine. This supports the hypothesis of Haddock that all bioluminescent Cnidarians require a dietary source of coelenterazine (Haddock et al. 2001). Adding coelenterazine to a non bioluminescent culture of *Obelia* was found to briefly restore its bioluminescence. Levels of the photoprotein obelin also dropped in cultures of *Obelia geniculata.* A lower level of coelenterazine was found in *Obelia longissima*, when compared to *Obelia geniculata* and *Obelia dichotoma.* Low levels of coelenterazine were also detected in *Myrina prolifer* which grows inside the *Obelia* colony and in *Chaetogammurus marinus* an amphipod commonly found living on the *Obelia* colonies. Three non luminous species of copepod present in the zooplankton were found to contain low levels of coelenterazine. The levels were not as high as those detected by Thomson in different species of deep water non luminous copepods (Thomson, 1995). These results are worthy of further investigation. Shimomura has previously found low levels of coelenterazine in other non luminous marine species (Shimomura, 1987). No coelenterazine containing bioluminescent copepods were found in a number of zooplankton trawls conducted in waters above *Obelia* colonies. In *Obelia geniculata* and *Obelia longissima* colonies ratios of obelin to apoobelin were found to change in older colonies at the end of the hydroid season, when compared to young colonies at the start of the season. As the colonies aged, the levels of apoobelin increased. The key question as to whether Obelia obtains coelenterazine through its diet or by biosynthesis remains

to be answered. A range of species were identified in zooplankton trawls, some of which are possible coelenterazine sources. Further work needs to be carried out to address this key question (refer to Section 9.2).

The problem of misidentification of Obelia species was solved. A protocol for distinguishing the three *Obelia* species found in Pembrokeshire was established. Fluorescence and bioluminescence were utilised to distinguish between them. They will also distinguish luminous species from taxonomically similar non luminous species. In addition it was also found that the habitat of an *Obelia* species is a significant factor in its identification. This led to the prediction that differences in photocyte patterns in different species could be caused by the hydroid trying to deter different predators from consuming different parts of its structure. Several species of predator were found living in and on *Obelia.*

GFP was recorded for the first time in the hydrotheca and tentacles of *Obelia dichotoma*.

Photek imaging results showed that no bioluminescence is produced in this part of the hydroid. This led to the hypothesis that GFP could be acting as a lure for prey or providing UV or temperature protection.

Utilising the chemiluminometer it was discovered that *Obelia longissima* has a lower mean bioluminescence than the other two Pembrokeshire species. This difference warrants further investigation. Using the Photek imaging system and analysis software, it was established that light emission from *Obelia geniculata* photocytes is longer (lasting over 60 seconds) than in *Obelia longissima* (main emission peaks produced within 20seconds). *Obelia longissima* patterns of

emission were mainly one or two rapid peaks, whereas *Obelia geniculata* showed multiple peaks of emission. Each photocyte appeared to be firing simultaneously. Obelia geniculata's emission patterns provided further evidence that calcium ions must be able to rapidly enter and leave the photocyte. Octanol was found to inhibit the bioluminescent response of *Obelia geniculata,* which supported the hypothesis of Dunlap et al that gap junctions exist between photocyte cell and support cells (Dunlap et al. 1987).

The potassium ion channel blockers tetraethyl ammonium chloride and 4 aminopyridine were found to produce a bioluminescent response in *Obelia geniculata*. This response was only seen at a relatively high concentration of 200mM.The hypothesis is that these chemical are blocking the potassium ion channels controlling the resting potential of the hydroids outer membrane. But equally they could be acting as chemical irritants to the hydroid.

The results of the protein experiments in chapter seven confirmed that in a polar solvent coelenterazine will produce a low level of chemiluminescence which increases with increasing concentrations of this luciferin. Human serum albumin will slightly increase this effect, but bovine serum albumin markedly increases coelenterazine chemiluminescence. Gelatin and human haemoglobin do not catalyse coelenterazine chemiluminescence.

Results confirm that albumin should not be used instead of gelatin in the coelenterazine assay. Albumin in human and animal blood will interact if luciferases are used in *vivo (*Vassel et al. 2012). The results supported the hypothesis that, "Bioluminescent proteins may have initially evolved as a solvent

cage consisting of a few key amino acids" (Vassel et al. 2012). In a classic luciferin-luciferase reaction the luciferase binds to the luciferin and will determine the colour of the light emitted.

"In fact all the luciferase does is create a solvent cage around the luciferin allowing one and only one oxygen atom in, and keeping water out (both oxygen & water quench electronically excited states" (Campbell, 2003b; Campbell, 1994; Watkins et al. 1993).Comparing sequence similarities did support Hastings's theory that "that bioluminescence must have evolved on at least 30 separate occasions" (Hastings, 1995).

Dual wavelength chemiluminometry confirmed that Obelia produces green rather than blue bioluminescence. A difference was found in the fluorescence maxima for Obelia geniculate (512nm) and Obelia dichotoma (518nm). This indicates that the fluorescence from Obelia geniculata is greener than expected. Results from dual wavelength chemiluminometry confirmed that bioluminescence produced from Obelia dichotoma and Obelia geniculate is nearly 100% green light rather than blue. This confirms that the GFP in Obelia photocytes acts as a fluor altering the wavelength of blue light produced by obelin. Photobleaching results illustrate the fact that wild type GFP in Obelia bleaches far slower than genetically engineered GFP in EGFP. This supports the hypothesis that Obelia has a molecular mechanism which protects its GFP from photo bleaching.

The photobleaching results also suggested that the photobleaching of GFP in *E.coli* may occur in two phases. The half-life for GFP bleaching in the first 6

minutes was longer than the half-life for the final 4mins, 2.56 ± 0.39 min compared to 1.01 mins.

Trial experiments in *Obelia dichotoma* suggested that the rate of photobleaching may vary, depending on the position of photocytes. More experiments need to be carried out to investigate this.

Half-lives of photobleaching of GFP could vary in different species of *Obelia*; this requires further investigation, together with the mechanism by which wild type GFP is protected from photobleachng.

9.2 Future studies

Further research could focus on the following areas:

9.2.1 The source of coelenterazine in *Obelia*.

Coelenterazine containing species of luminous copepod remain a possible dietary source of luciferin for bioluminescent hydroids, more zooplankton trawls, at varying depths need to be carried out to ascertain if these are present at Pembrokeshire *Obelia* sites. The coelenterazine assay needs to be utilised to quantify coelenterazine levels in other species of zooplankton already identified in trawls. The reason that *Obelia longissima* has a lower bioluminescence needs further investigation; this could be linked to lack of coelenterazine in its diet. Milford Haven marina zooplanktons need to be tested for coelenterazine content. Equally the salinity of *Obelia longissima's* habitat could influence its bioluminescence or the lower number of its photocytes. Biosynthesis of coelenterazine in *Obelia* remains a possibility. This hypothesis could be tested by

combining an *Obelia* homogenate with the tripeptides required for coelenterazine synthesis. More culture experiments could discover the factors which effect bioluminescence in *Obelia,* which could contribute to overall knowledge. The genes controlling coelenterazine synthesis need to be identified. Levels of obelin, apoprotein, coelenterazine and bioluminescence could all be monitored in cultures. If the temperature of the Milford Haven waterway rises, either due to climate change or the predicted effects of the new power station, bioluminescence of *Obelia* colonies may alter.

9.2.2 Fluorescence in *Obelia*

The presence of GFP in the hydranths of *Obelia dichotoma* warrants further investigation. The hypothesis is that this is acting as an UV block or lure for prey. FLIM analysis could be utilised. The key question as to whether the rate of photobleaching of GFP varies in different species of *Obelia* also requires further investigation. The prediction is that photobleaching is prevented by coelenterazine acting as an oxygen scavenger (Rees et al. 1998). Coelenterazine's role as an "antioxidant" is linked to the theory that it originally evolved as an oxygenase enzyme. Further investigations into the "solvent cage theory could be conducted". Alternatively GFP in a dimer form could withstand photobleaching.

Cnidarians can survive without fluorescence or bioluminescence, but the selective advantage of these light emissions needs to be established. The hypothesis of AK Campbell is that GFP protects against an increase in temperature. Fluorescent patterns of the two possible species of *Obelia's* medusa need investigating to establish if they are identical.

9.2.3 Studies on ion channels and patterns of light emission

The m-RNA could be extracted from the hydroids and probed for sequences which would indicate the presence of certain ion channels in the hydroids membrane. PCR could be utilised to ascertain if the message for particular channels is present. Western blot analysis would confirm if the message is being converted into real protein. Further experiments could be carried out on all three species using a range of ion channel blockers. An OAS analysis needs to be carried out on *Obelia dichotoma* to establish if it also has a different pattern of bioluminescent light emission. The effect of 4 aminopyridine on bioluminescence needs more investigation. A wider range of dilutions of 4 aminopyridine could also be used. It would also be worthwhile soaking hydroids in a blocker before trying to trigger bioluminescence with potassium ions. These results would help to answer the key question as to how bioluminescence is triggered in *Obelia*.

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Appendices

Publication relevant to this thesis

Vassel N, Cox CD, Naseem R, Morse V, Evans RT, Power RL, Brancale A, Wann KT and Campbell AK (2012).Enzymatic activity of albumin shown by coelenterazine chemiluminescence. Wileyonlinelibrary.comDOI 0.1002/bio.2357.

Presentations/posters given

Presentation/abstract title	Scientific meeting
"What makes seaweed sparkle?"	Opening doors to science. Darwin Centre for Biology & Medicine. Haverfordwest Pembs
The regulation of bioluminescence in *Obelia geniculata.*	Hydrozoan Society meeting MBA Plymouth
Bioluminescence in *Obelia geniculata.*	Cross border research seminar. Welston Court Science Centre. Milton, Pembs.
Bioluminescence in hydroids. (Poster)	"Science in the bay" Cardiff Bay, science event.
The regulation and origin of bioluminescence in *Obelia.*	Cell physiology research group seminar. Welston Court Science Centre. Milton, Pembs.
"Lighting up your thesis". Interesting the public in marine science.	Public Engagement Student Showcase. Cardiff University.

Training courses attended

Course title	Venue
DNA techniques (3 days).	Medical School Cardiff University
Practical PCR and bioinformatics (5days).	Institute of Technology Carlow
Beginning your PhD.	Graduate centre Cardiff University
Finding electronic information.	Graduate centre Cardiff University
Plankton Trawling and identification (8 days total).	Dale Fort field Studies centre.
EMBO advanced optical microscopy course.	MBA Plymouth.