Visualisation of bioluminescent *Vibrio midae* SY9 K811 in juvenile *Haliotis midae* abalone using non-invasive bioluminescence imaging

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List of Abbreviations

The following abbreviations have been used in this thesis.

3’	three prime
5’	five prime
α	alpha
λ	lambda
°C	degree Celsius
µ	micro
µg	microgram (s)
µl	microliter (s)
µM	micromolar
μ_{max}	maximum specific growth rate
%	percentage
Amp	ampicillin
bla	
bp	base pair(s)
cat	chloramphenicol acetyltransferase
CCD	Charged couple devise
cfu	colony forming units
Cm	chloramphenicol
Col	colony
dH_2O	deionised water
DNA	deoxyribonucleic acid
dNTP	deoxynucleosidetriphosphate
EDTA	ethylenediamine tetraacetic acid
FOV	field of view

g	gram(s)
g	gravity constant (9.81 ms-1)
GFP	green fluorescent protein
GIT	gastrointestinal tract
hr	hour(s)
H_2O_2	hydrogen peroxide
IVIS	*in vitro* imaging system
kb	kilobase(s)
l	litre(s)
LB	Luria Bertani broth
LED	light emitting diode
m	milli
M	molar
MCS	multiple cloning site
$MgCl_2$	magnesium chloride
min	minute(s)
ml	millilitre(s)
mm	millimetre(s)
mM	millimolar
NaCl	sodium chloride
NADPH	nicotine adenine dinucleotide phosphate (reduced form)
ng	nanogram (s)
nm	nanometer (s)
NTC	non template control
OD	optical density
p/s	photons per second
PCR	polymerase chain reaction
pH	negative decimal logarithm of the H^+ concentration
pmol	picomol

rpm	revs per minute
ROI	region of interest
SDS	sodium dodecyl sulphate
sec	second(s)
Sm	streptomycin
SSS	sterile sea salts
TAE	tris-acetate EDTA buffer
TSA	Tryptic Soya Agar
TSB	Tryptic Soya Broth
U	unit(s)
V	Volt
v/v	volume per volume
w/v	weight per volume

Abstract

Bioluminescence imaging (BLI) is a powerful technique that enables real time monitoring of various processes such as gene regulation, host-pathogen interaction and distribution of bacterial cells *in vivo*. Most of these processes however have only been monitored in murine models or mammalian tissue culture systems. In order to determine whether BLI could be used to monitor bacterial-host interactions in an invertebrate system, the probiotic bacterium *Vibrio midae* SY9 was genetically labelled with a bioluminescent plasmid (pKluxCat) containing the *lux* operon from *Photorhabdus luminescens.* The plasmid was modified by the addition of the Tn9 chloramphenicol acetyltransferase (*cat*) gene to enable selection of *Vibrio* transconjugants. The bacterial cells were labelled using a conjugal mating technique and the resulting transconjugant cells were highly bioluminescent with a minimum of 5.264 $x10^4$ colony forming units detectable in liquid culture. The bioluminescent cells emitted light stably and consistently without compromising the growth of the strain, and bioluminescence emission was at its highest during the logarithmic growth phase. A strong linear correlation between bioluminescence emitted and bacterial numbers (r^2=0.99) was found, indicating that the amount of bacteria present *in vivo* could be accurately quantitated using bioluminescence. Administration of the bioluminescent bacterial cells by oral gavage to juvenile *Haliotis midae* abalone allowed non-invasive visualisation of bioluminescence within abalone for up to 10 hours post-gavage. This study therefore demonstrates the potential for using bioluminescence imaging to investigate the interaction of bacteria with an invertebrate host.

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Chapter 1:

Literature Review

1.1 Status of global aquaculture

Aquaculture of fish and shellfish has been carried out for centuries, owing its success to a rapidly expanding human population, increasing consumer demand for selected seafood products and diminishing wild-stocks. Aquaculture is defined as the rearing or breeding of aquatic animals, fresh water and marine, for all or part of their life cycles in a controlled environment (FAO, 2009). The intensification of aquaculture and globalization of the seafood trade has led to remarkable developments in the global aquaculture industry (Wang *et al.*, 2008), which has expanded significantly over the past 50 years. In 2010, aquaculture practises contributed 148.5 million tonnes of product with an estimated net value of 119.4 million US dollars (FAO, 2010). Among the organisms cultured aquaculture of molluscs account for 21% of total aquaculture products and accounts for 65% of total molluscs produced when captive fisheries are taken into account (Kesarcodi-Watson *et al.*, 2008).

1.2 Abalone aquaculture in South Africa

Abalone aquaculture is by far the most lucrative aquaculture sector in South Africa. This aquaculture practice represents approximately 94% of the entire sector in terms of monetary value (DAFF 2012), and in 2006 South Africa was the largest producer of cultured abalone outside of Asia (Troell *et al.*, 2006). The market opportunities for abalone are numerous, especially in countries like Japan, China and North America where demand continues to grow for abalone products, both wild and cultured, and new sources of supply are always of great interest (Oakes & Ponte, 1996). There are five endemic species of *Haliotis* in South Africa (Branch *et al.*, 1994) of which only *Haliotis midae* is of commercial interest. Initially the local aquaculture industry was reluctant to invest in abalone aquaculture, mainly due to the slow growth rate of *H. midae*. However,

subsequent studies demonstrated that this species could be successfully spawned and reared in captivity (Genade *et al.*, 1988) and that *H. midae* grown in captivity had a much faster growth rate compared with animals grown in the wild (Hahn K.O, 1989). Another deciding factor for the establishment of the industry was a new market for "cocktail" sized abalone (~100g). Wild harvested abalone need to be significantly larger due to the laws and regulations in place to protect this species in South Africa, and these animals take approximately 12-14 years to reach legal size. The cocktail sized abalone however, takes between 4-5 years to reach the 100-120g mass required for sale of these animals. These findings, coupled with the rapid depletion of wild abalone stocks as a result of over-fishing, poaching and destruction of their natural environments, led to renewed interest in the cultivation of *H. midae*.

To date, the cultivation of abalone in South Africa has been a huge success and the high demand and market prices fetched for abalone meat and products has demonstrated the economic viability and potential of abalone farming in South Africa (Troell *et al.*, 2006). Access to cheap labour, favourable coastal water quality and infrastructure has also facilitated rapid growth of the local abalone industry. Presently, there are more than 14 farms that exist along the South African coastline (mostly pump-a-shore farming operations) producing in excess of 1,000 tonnes of abalone per annum with an estimated value of R 355 million in 2010 (DAFF, 2012).

1.3 Bacterial microorganisms in aquaculture

Aquatic environments are a rich source of bacterial organisms which can be categorized as being opportunistic, beneficial or neutral (Sultze *et al.*, 2006). The bacterial and animal interaction in an aquatic environment is close, in that animals are essentially bathed in bacteria, and the bacteria that exist in the environment are also in direct contact with mucosal surfaces of gut and gills. For most aquatic species, eggs are released directly into the environment so when larvae hatch, they are immediately exposed to the microbial population present in the surrounding environment before their immune system has had a chance to develop. This would include the mucosal barrier of the digestive tract (Verschuere *et al.*, 2000). It then stands to reason that the

microbial population within the aquatic environment is very important as it plays a direct role in the early development of larvae.

1.3.1 Pathogenic bacteria

Many aquaculture operations, particularly hatcheries, strive to maintain a clean and pathogen free environment by implementing strict biosecurity measures, sterilising equipment and treating water sources. Water is often treated with filters, ozonation or UV light to eliminate bacteria (Sultze *et al.*, 2006). Complete elimination of bacteria is however, almost impossible as microorganisms are very easily introduced into these systems via natural and artificial food sources, incoming water (Hansen & Olafsen, 1999; Sandaa *et al.*, 2003), equipment used for cleaning and maintenance of systems as well as the day-to-day handling of animals by farm personnel. Although these biosecurity measures are implemented to encourage the maintenance of healthy animals, the loss of natural microorganisms associated with these animals often encourages the proliferation of opportunistic pathogens that make it through the disinfection process. Aquaculture is an intensive practise, with animals cultured at high densities and often fed large amounts of feed to promote rapid growth of the cultured organism. As a result, the use of sub-optimal feeds and poor management practices (over feeding, or failure to remove excess feed) can rapidly deteriorate water quality and promote the proliferation of opportunistic/ pathogenic bacterial strains (Olafsen, 2001). Abalone, as with most other aquatic organisms, have a higher rate of interaction with microorganisms in their immediate environment compared with most terrestrial animals, potentially making them more susceptible to invasion by opportunistic pathogens.

1.3.2 Antimicrobials

Diseases are recognised as a significant restriction on aquaculture production and trade, and have affected the economic development of the sector in many countries (Verschuere *et al.*, 2000). Until recently, broad-spectrum antimicrobial substances were routinely used as a preventative measure and for treatment of infected animals (Balcázar *et al.*, 2006). Excessive use of broad-spectrum antimicrobials has however

resulted in the emergence of antibiotic resistant bacterial strains (Gomez-Gil *et al.,* 2000; Olafsen, 2001, Bansemir *et al.*, 2005). In Asia, excessive antibiotic usage has had a huge impact on the shrimp industry, resulting in large scale production crashes due to the emergence of resistant strains (Kesarcodi-Watson *et al.*, 2008). More recently, the use of antibiotics in food production has been strictly regulated due to the numerous adverse effects of these drugs. Some of these effects include immunosuppression in the treated organsm, destruction of environmental microbial flora (Yousefian & Amiri, 2009) and interrupted intestinal development in larvae (Prado *et al.*, 2010). Furthermore, it has been suggested that the accumulation of antibiotics in animal tissues may lead to the spread of antibiotic resistance to human pathogens, posing a significant risk to human health (Witte 1997, Verschuere *et al,.* 2000, Kesarcodi-Watson *et al.*, 2008, Prado *et al*., 2010). As a result, there is increased interest within the industry to control or completely eliminate the use of antimicrobials (Gomez-Gil *et al.*, 2000) and to seek alternate measures for the treatment and control of disease outbreaks in animal production. This has resulted in a significant interest in the field of probiotics.

1.3.3 Probiotic bacteria

Probiotics are defined as live microbial feed supplements that beneficially affect the host animal by improving its intestinal balance (Fuller, 1989). This definition was later revised by Verschuere *et al*. (2000) who defined probiotics as live microbial adjuvants, which have beneficial effects on the host by ensuring improved use of feed or enhancing its nutritional value, by enhancing the host's response towards disease or by improving the quality of its natural environment. In essence probiotics should protect against pathogen proliferation, help in the digestion of feed, improve water quality and stimulate the immune system (Macey, 2005). The possible modes of action of probiotics include: production of inhibitory compounds, competition for nutrients or available energy, competition for adhesion sites, enhancement of the immune response, improvement of water quality, provision of a source of micro or macronutrients and lastly, provision of enzymes to facilitate feed digestion (Verschuere *et al*., 2000)

The selection criterion for isolating a potential probiotic has been very clearly defined. Some important characteristics that a potential probiotic should possess before it is utilised in farm scale trials include: the ability to outcompete pathogens; the probiotic strain should be able to reach the target area where its effect is to be exerted and establish itself there and, it should ultimately be able to establish itself within its host. These characteristics are essential so that probiotics may serve as a measure of protection (production of antimicrobials and antifungal compounds, immunostimulation) when the host has been challenged with a potential pathogen (Gomez-Gil *et al.*, 2000, Verschuere *et al.*, 2000; Balcázar *et al.*, 2006; Kesarcodi-Watson *et al.*, 2008; Prado *et al.*, 2010). Of the characteristics mentioned above, the ability of the potential probiotic to establish itself or colonise the gastrointestinal tract of its host is very important. It is therefore essential to evaluate the persistence of the probiotic in the digestive tract of its host in order to evaluate its probiotic potential (Macey & Coyne, 2005a).

Abalone are slow growers, and production costs (feed, electricity, labour, water system upkeep) for maintenance of these animals are quite high. In order to maximise profits, and keep the abalone aquaculture industry sustainable in the global market, farmers are investigating ways to improve growth rates and reduce mortalities, hence the investigation into probiotics for abalone. However, the price demanded for premium abalone products has risen steadily, creating an economic environment in which abalone aquaculture is becoming increasingly attractive as a financial investment (Gatesoupe, 1999) and this too may have fuelled the investigation into the use of probiotics for maintenance of healthy cultured animals.

1.4 Methods for detection and enumeration of bacterial populations

Numerous techniques exist for the detection and quantification of microorganisms within the environment and animal tissues, and each method has certain advantages and disadvantages (Greeff *et al.*, 2012). Commonly used methods include viable cell counts and phenotypic identification (Li *et al.*, 1996; Harmsen *et al.*, 1999; McCartney, 2002); immunological assays, such as enzyme-linked immunosorbent assay (ELISA) and immunocolony blot (Spanggard *et al.*, 2000, Cunningham, 2002); and genetic

techniques, such as *in situ* hybridisation (McCartney, 2002; Macey and Coyne, 2005b; Iehata *et al*., 2009), polymerase chain reaction (PCR) (Babalola, 2003) and ribosomal RNA gene sequencing (Li *et al.*, 1996). Of the techniques mentioned above, viable cell counts, *in situ* hybridisation and ribosomal RNA gene sequencing are the most commonly utilized methods for detection of microorganisms.

1.4.1 Direct methods of bacterial detection and quantification

1.4.1.1 Viable cell counts

Viable cell count, determined by inoculating bacterial and/or fungal samples on selective or differential media, is the simplest form of isolation and enumeration. This technique relies on culturing of microorganisms before identification is made using biochemical tests for phenotypic characterisation. The most notable disadvantage of this technique is that less than 10% of microbes are culturable, even if they are viable (Billard & DuBow, 1998), and this means that isolating bacterial species from an organism or environmental source for example, does not always provide a true representation of the bacterial strains present. In some cases, colony counts are quite biased and show poor reproducibility as bacterial numbers and species isolated depend on how samples are obtained, treated and on which selection medium they are inoculated. Furthermore, subsequent phenotypic characterisations are often laborious, time consuming and inaccurate, and the difference in observed numbers of bacteria or their phenotypic characteristics are often influenced by individual investigator bias (Kepner & Pratt, 1994). Another source of error is that bacterial species that have been isolated from the same source often appear very similar in appearance, making phenotypic separation of the strains quite difficult (Harmsen *et al*., 1999; McCartney, 2002). Consequently, culture based techniques are not sensitive enough and often underestimate or fail to detect the target organism. However, culture based techniques have been widely utilised to great effect, especially when strains have been labelled by the addition of a gene that confers resistance to an antibiotic (such as the *bla* gene coding for resistance to ampicillin) and plated on selective media.

1.4.2 Indirect methods of detection and quantification

1.4.2.1 *In situ* hybridisation

In situ hybridisation (ISH) is one of the most widely utilised indirect techniques for detection and quantification of environmental bacteria and is often employed for assessing bacterial colonisation within an animal host. This technique involves the use of synthetically prepared oligonucleotide probes which hybridise to a specific DNA or RNA sequence of the targeted bacterial strain. This technique can be extremely sensitive, if optimized correctly.

Fluorescent *in situ* hybridisation incorporates the use of a fluorescent probe which makes direct visualization and enumeration of the target organism or gene possible (McCartney, 2002; Namsolleck *et al.*, 2004, Koo *et al*., 2006). *In situ* hybridisation and fluorescent *in situ* hybridisation have both been used for probiotic studies (Tanaka *et al*., 2004; Macey & Coyne 2005b, Iehata *et al*., 2010) and these techniques have enabled the detection of bacteria within the digestive tract of the host organism. Approximately 10 to 50% of gut bacteria are culturable (Zoetendal *et al*., 1998), therefore using culture based techniques to enumerate or monitor gut bacteria is not always an option as the microorganism may be viable, but unculturable (Billard & DuBow, 1998). The major advantage of this technique is that it does not require the bacterium to be cultured. This technique does not discriminate between culturable and unculturable bacterial cells, therefore providing a precise representation of the number of bacterial cells present in the gut. The disadvantage of this technique is that it can be hindered by restrictions of cost, time and technical requirements (Namsolleck *et al*., 2004).

1.4.2.2 16S rRNA sequencing

Sequencing the 16S rRNA gene is another frequently used technique for bacterial identification. The small-subunit rRNA (16S), is a component of the 30S small subunit of prokaryotic ribosomes, and is commonly used as a target for probes as these ribosomal subunits are abundant and have evolutionary information that enables identification of close as well as distant phylogenetic relationships (Li *et al*., 1996; Mignard & Flandrois, 2006;). The 16S rRNA gene contains conserved sequences which can serve as a template

for PCR primer design and as a result, universal 16S primer pairs are available to amplify up the various conserved regions (Babalola, 2003). In addition to the conserved regions, the 16S rRNA also contains hyper-variable regions that allow for species specific identification of bacteria (Kolbert & Persing, 1999). With increased availability of sequencing facilities and decreasing costs for high throughput sequencing operations, determination of the 16S rRNA gene sequence has emerged as a viable option for strain identification to genus or species level (Li *et al.*, 1996; Mignard & Flandrois, 2006).

Although the molecular techniques discussed above have proven very useful for bacterial identification and more specifically for studies which have involved investigation of the colonisation potential of bacterial strains (Macey, 2005; Huddy, 2010), the one major downfall of all of these techniques is that they require the host animal to be sacrificed. This makes repeated sampling from the same individual almost impossible and the collection of time series data then requires a large number of animals in order to obtain statistically relevant data.

1.5 Non-invasive imaging

Monitoring biological processes *in vivo* would greatly enhance our understanding of how various biological processes function in real time. Conventional imaging of animals often required them to be sacrificed before the tissue or organ was dissected and stained to identify the location and state of a particular molecule at a specific point in time (Meighen, 1993). Over the years there has been a movement towards the use of non-invasive imaging methods which enable processes such as gene expression to be visualized in living animal tissues. An emerging field termed 'molecular imaging' employs a variety of imaging modalities which allows for visual representation, characterisation and quantification of biological processes with intact organisms (Contag & Bachmann, 2002; Wiles *et al.*, 2006). Molecular imaging methods for detection of various processes are favoured, as the instrumentation used reduces or completely eliminates the need for animals to be killed, thereby allowing extensive monitoring of each animal over the entire duration of an experiment (Contag & Bachmann, 2002; Contag & Ross, 2002). Each animal serves as a biological repeat so

that the data obtained is more statistically accurate and possibly provides a better understanding of the molecular mechanism under investigation.

Well established imaging modalities used by some researchers include position emission tomography (PET), single photon emission tomography (SPECT), magnetic resonance imaging (MRI) and optical imaging (Contag & Ross, 2002; Baker, 2010). These techniques allow visualisation deep within tissues and each method has its pros and cons (Table 1) (Lyons, 2005; Koo *et al*., 2006; Baker, 2010) so that their use in animal experiments is largely dependent on the process under investigation. PET, SPECT and MRI are often used for whole body imaging and disease detection in humans, but smaller less expensive machines have been manufactured specifically for animal research (Baker, 2010). Even though these technologies are routinely used in animal research, PET and SPECT utilize radiolabelled probes for imaging, which is unfavourable due to the potential health risks. Furthermore, these techniques require the use of specialised protective clothing, facilities and training, which means the use of these technologies can be quite costly. Optical molecular imaging on the other hand does not require the use of radiolabelled probes.

1.5.1 Optical Molecular Imaging

Optical imaging is a new and rapidly developing technology that employs the use of optical probes for tracking and reporting functional information regarding molecules, proteins and cells *in vivo* (Troy *et al.*, 2004). Advances made in optical imaging have provided valuable insight into biological processes within intact cells and small animal models (Badr & Tannous, 2011) and has enabled researchers to follow processes that could previously not be detected or monitored. This method of imaging is also quite popular because optical instruments are about a quarter of the cost of other imaging technologies and the instruments are easy to use (Baker, 2010). The advantages of this method of imaging include shorter imaging times, it does not require specialized personnel and more than one animal can be imaged at a time (Contag & Bachmann, 2002, Chuang & Cheng, 2010). A critical component of optical imaging modalities are sensitive detectors based on charge coupled device (CCD) cameras. Extremely low

levels of light can be detected using CCD cameras and, because they allow real time visualisation and quantification of a signal, optical imaging techniques can be used to study the physiological response of single cells as well as spatial distribution of markers on a surface of or within a tissue (Billard & DuBow, 1998).

Optical machines such as the Lumina range by Xenogen (Calliper life science) comprises a CCD camera mounted in a light sensitive chamber. For certain models, the CCD camera can be cooled to temperatures as low as -120 °C. The drop in temperature reduces thermal noise, thereby greatly improving signal-to-noise ratios, while preserving the spectral sensitivity of the CCD (Contag & Bachmann, 2002). These specifications essentially allow for sensitive detection of even the smallest amount of light emitted from a sample or organism and images obtained are of a higher quality. Optical imaging protocols usually employ the use of light emitting probes or marker genes which include fluorescent and bioluminescent constructs or markers.

Table 1. Advantages and disadvantages of optical imaging methodologies used to detect biological processes in live animals.

Technique	Radiation spectrum	Resolution	Acquisition time	Advantages	Disadvantages
PET	High energy gamma rays	1 - 2 mm	Minutes	→High sensitivity →Variety of probes available →quantitative	→Cyclotron needed to generate short lived radio isotopes → Low resolution → unincorporated substrate can increase noise
SPECT	Low energy gamma rays	1 - 2 mm	Minutes	→Imaging of multiple probes simultaneously → Radioisotopes have a longer half-life than those used in PET	→ 10 to 100 fold less sensitive than PET →Requires radiation dose to be administered to animals
MRI	Radiowaves	25 -200 mm	Minutes to hours	→ Provides both anatomical and functional detail → No exposure to radiation	→ Low sensitivity → Long image acquisition and processing times →High instrumentation cost
Optical Bioluminescence Imaging (BLI)	Visible light	1 - 10 mm	Minutes	→ Highest sensitivity → No external light stimulation required → Low cost → No radiation exposure	→ Low resolution → 2-D image output →Requires genetic modification of cells or animals → Light diminishes with increased tissue depth
Optical Fluorescence Imaging	Visible light or near infrared light	1 - 10 mm	Seconds to minutes	→High sensitivity → Multiple reporter wavelengths can be measured → Low cost	→ Low resolution → Autofluorescence of non-labelled cells increases noise → Light diminishes with increased tissue depth

1.5.2 Optical imaging using fluorescence

Optical fluorescent imaging involves the use of green, yellow and red fluorescent proteins (GFP, YFP, and RFP). A unique feature of these molecules is that they naturally fluoresce when their fluorophores are excited to a higher energy level under light illumination (Troy *et al*., 2004; Macey & Coyne, 2005b). GFP, the most commonly used fluorescent protein, was first isolated from the bioluminescent jellyfish *Aequorera aequorin* (Chuang & Cheng, 2010). It was then cloned into *Aequorera victoria* where it was noted that light was produced when calcium ions bound to the jelly fish aequorin photoprotein. The photoprotein is then able to activate a second protein, a green fluorescent protein, which is the source of fluorescent light emission in the jellyfish (Billard & DuBow, 1998). Characterisation of the *gfp* gene and its expression in prokaryotic and eukaryotic systems has demonstrated the protein's ability to be used as a reporter of gene expression and protein localisation in living cells (Billard & DuBow, 1998). The one major advantage of using a GFP reporter is that it does not require the addition of exogenous substrates; fluorescence can simply be generated by exposure to long ultraviolet wavelengths (450 to 650nm light spectrum) (Billard & DuBow, 1998). Various versions of GFP have been described over the past few decades, including a mutated GFP (contains histidine in place of Tyr-66) that produces blue fluorescence (Heim *et al*., 1994; Billard & DuBow, 1998). A newly discovered RFP (DsRED) emits light at 583nm, which is lower than the emission wave length for cellular autofluorescence, and therefore produces less background interference than GFP (Hakkila *et al.*, 2002; Chuang & Cheng, 2010). Because these fluorescent proteins emit light at different wavelengths, the difference in the emission spectrum enables fluorescent detection over a wider range, therefore making it possible to image different types of molecules of interest in tissue and organs.

Fluorescent markers are commonly attached to bacterial strains in order to track their progression in disease models or monitor their presence in various environments or tissues. Because these proteins do not require the addition of exogenous substances to function, this technique has proven to be quite useful for monitoring tagged bacteria, as bacterial presence can be detected at a faster rate, compared to the time taken to enumerate bacterial numbers using colony counts. This was apparent in a study carried out by Leff and Leff (1996) who noted that a GFP tagged *E. coli* stain was more readily

detected by the bacterium's ability to florescence than by screening for the microbe using plate counts. Once a bacterial cell, labelled with a fluorescent marker, synthesises the fluorescent proteins, the protein will remain functional and a signal will still be detectable even after the bacterial cells are no longer viable, allowing for the detection of non-culturable bacteria (Hakkila *et al*., 2002; Sagi *et al*. 2003). This could however also be regarded as a disadvantage because it could potentially give a false indication of viable cells.

Fluorescent proteins such as GFP have been used in *in vivo* animal imaging. This was demonstrated in a study where mice were infected with a GFP labelled *E. coli* strain in order to determine and monitor the process of infection, the tissue specificity and spatial migration of the infectious agent (Zhao *et al*., 2001). The aforementioned researchers were able to demonstrate the localisation of the tagged bacteria using the light emission properties of the GFP protein and CCD technology. One of the disadvantages of *in vivo* animal imaging is that the tagged cells have to be illuminated with an external light source in order to fluoresce. Therefore, light has to travel through the skin and tissue of the animal resulting in scattering or absorption of light by the tissue before it is detected. Another disadvantage of using GFP in *in vivo* imaging is the autofluorescent nature of some mammalian and most marine organisms. Autofluorescence is the single most important source of background in fluorescent imaging experiments and this has a significant effect on fluorescence detection (Contag & Bachmann, 2002). The amount of autoflourescence can be so extensive that the fluorescence emitted by the GFP labelled molecules under investigation is completely masked. As a result, it often leads to the acquisition of poor quality images which could result in incorrect interpretation of experimental results. Consequently, researchers have now begun to investigate alternative optical probes for *in vivo* imaging and this has given rise to the field of bioluminescent optical imaging.

1.5.3 Optical imaging using bioluminescence

Bioluminescent imaging is a technique that relies on the detection of photons emitted by cells or tissue of living organisms (Badr & Tannous, 2011). It centres on a phenomenon known as bioluminescence which is a natural process that involves the

emission of a photon of light in a reaction catalysed by the activity of a luciferase enzyme. Bioluminescence has been observed in many different organisms including bacteria, fungi, fish, insects, algae and squid (Meighen, 1993). Of the organisms mentioned, bioluminescent bacteria are the most widely distributed luminescent organisms in nature, and their luciferase system is one of two that is predominantly used in research (Billard & DuBow, 1998).

Luciferases have been used as reporter genes and sensors for decades. Studies have included evaluation of gene expression in cultured cells, analysis of circadian rhythms and whole body imaging of rodents (Contag & Ross, 2002). Production of light or luminescence in naturally occurring bioluminescent bacteria is encoded by five genes that are organised in an operon, *lux*CDABE, which is referred to as the lux operon. Bacterial luciferase (product of the *lux*AB genes), catalyses the oxidation of reduced flavin mononucleotide ($FMNH_2$) and a long chain fatty aldehyde (coded by the *lux*CDE genes) with oxygen, resulting in the emission of green/blue light at 490nm (Meighen, 1993; Billard & Dubow, 1998) (reaction shown below).

$$\mathbf{FMNH_2 + O_2 + RCHO \rightarrow RCOOH + H_2O + Light\ _{(490nm)}}$$

The bacterial *lux* operon is the only bioluminescent system that does not require the addition of an exogenous substrate to function, as the genes that encode the substrate synthesising enzymes are present within the operon. The fact that exogenous substrates do not need to be added to luminescent reactions, coupled with the ease at which the genes can be transferred to non-luminescent bacteria, has made it a useful biomarker. The bacterial *lux* system therefore acts as a light source that can be used to monitor gene expression as well as distribution of a bacterial species. One of the reasons why there has been a marked interest in using bioluminescent reporters for *in vivo* imaging is because there are very few naturally luminescent organisms. This means that the chance of encountering autobioluminescence is unlikely, reducing the likelihood of background luminescence affecting the outcome of an experiment. This is of critical importance for marine organisms which usually have a high autofluorescent nature.

The bioluminescent signal emitted by the bacterial *lux* operon has been an ideal marker for studying pathogen-host interactions, as well as for determining bacterial viability within living organisms to which these labelled bacterial strains have been administered. Since the reaction requires $FMNH_2$ and oxygen to proceed, which is only available in actively growing cells, no signal would be detected when these molecules are not available. This was demonstrated in a study carried out by Francis *et al.* (2000) who used bioluminescent modified *Staphylococcus aureus* strains to infect mice in order to show that the labelled bacteria could be detected *in vivo* without sacrificing the animals. They also used it to monitor the effectiveness of the antibiotics administered to these mice by looking at the number of viable cells after treatment. A decrease in the bioluminescence signal eight hours post infection was noted, and it was concluded that the decrease in the bioluminescence was due to the antibiotic treated mice clearing the infection. The signal was completely lost 24 hours post infection, indicating full clearance of the infectious agent. The opposite was noted for the untreated control mice which had a strong bioluminescent signal after 24 hours, indicating that infection was still rife and that the bioluminescent labelled *S. aureus* cells were still viable.

The advantages of using a bioluminescent marker system and therefore bioluminescent imaging are numerous. Firstly, the ability to clone and transfer genes to almost any biological system greatly increases the scope of its use (Meighen, 1993). This system also allows non-invasive monitoring of various processes, such as disease progression and bacterial colonisation, whereby bioluminescent bacteria, whether a pathogen or probiotic strain, can be monitored as it migrates to various tissues (Wiles *et al.*, 2009). This non-invasive method is safer than other imaging modalities and at the same time cost effective, because as mentioned before, the instrumentation is far less expensive compared to other imaging platforms. Another advantage is that fewer animals are sacrificed and repeated measures can be taken from the same individual animal which greatly enhances the significance or statistical power of the data collected. This system also allows rapid monitoring of bacterial loss or proliferation in a shorter time than it would take to enumerate cell numbers using a plating technique (Meighen, 1993). Studies conducted have demonstrated that bioluminescence detection (bioluminescence signal measured), both in culture and in animals, has corresponded to bacterial cfu (colony forming units) data with a correlation coefficient of 0.98

(Rocchetta *et al*., 2001), therefore making this technique very sensitive and highly accurate.

The disadvantages of using a bioluminescent system in *in vivo* animal imaging is that the intensity of the light produced by intact cells is dependent on the concentration of substrate available (Wilson & Hastings, 1998). Light emission by the lux operon requires energy and oxygen from cell metabolism, therefore the metabolic state of cells can affect the bioluminescent signal detected. As metabolism decreases, the amount of substrate available becomes limited, ultimately decreasing the amount of bioluminescence produced. Another problem with the luciferase system is the wavelength dependant transmission of light through animal tissue as it has been stated that there is approximately a 10 fold loss of photon intensity for each centimetre of tissue that light has to pass through (Sadikot & Blackwell, 2005). Consequently, although bioluminescence imaging is a unique and powerful optical imaging tool, quantitative analysis must be approached with caution, and validation of applications may be necessary (Wilson & Hastings, 1998).

1.6 Rationale and aims of this study:

Abalone farming is a lucrative practise in South Africa and the exportation of abalone and abalone products greatly contributes to the South African economy. The slow growth rate of abalone and the increased susceptibly of these animals to emerging diseases are however two factors that are hampering further growth and development in this industry. Probiotics are regarded as a viable alternative to the use of broad-spectrum antimicrobials for disease prevention as well as improved growth and nutrition of cultured abalone. Indeed, Macey and Coyne (2005) demonstrated that dietary supplementation with the probiotic bacterium *V. midae* SY9 increases the growth rate and disease resistance of farmed abalone and further identified the potential for *V. midae* SY9 to colonise the gastrointestinal tract of these animals (Macey 2005, Huddy & Coyne, 2014). Various techniques were employed to demonstrate localisation of strain SY9 within the crop/stomach and intestinal region of the abalone digestive tract, however both studies required sacrificing the animals (Macey, 2005;

Huddy & Coyne, 2014). As mentioned earlier, molecular imaging has become a viable means of monitoring bacterial colonisation through the use of fluorescent and bioluminescent constructs. This imaging method allows real time monitoring of bacterial movement without having to sacrifice the animal host. For this study, the use of a GFP-labelled bacterium was not ideal due to the high autofluorescent nature of the abalone shell (Proudfoot *et al*., 2008). Sandenbergh and Roodt-Wilding (2012) also found juvenile *H. midae* abalone to be highly autofluorescent, with autofluorescence predominantly prominent at the same wavelengths as that of GFP. Consequently, the bacterial *lux* operon (*lux*CDABE), which confers a bioluminescent phenotype to the host, was investigated as a reporter of bacterial cell viability because both *V. midae* SY9 and *Haliotis midae* are non-bioluminescent. *In vivo* monitoring of bioluminescence was first developed using a gastrointestinal infectious disease model, where a species of *Salmonella typhimurium* transformed with the *P. luminescens lux* operon*,* thus constitutively expressing luciferase*,* allowed the tissue distribution and virulence of various *S. typhimurium* strains to be monitored *in vivo* (Contag *et al.*, 1995, Contag & Bachmann, 2002; Doyle *et al.,* 2004). Bioluminescent *E. coli* transformed with the *P. luminescens lux* operon also allowed *in vivo* tracking of the effectiveness of antimicrobial agents in the neutropenic mouse thigh model (Rocchetta *et al*., 2001*).* These studies demonstrated that *in vivo* bioluminescence imaging, using the *P. luminescens lux* genes, was feasible and could be used to quantitatively assess experimental data (Contag *et al.,* 2005).

The aim of this study was therefore to investigate the use of bioluminescence imaging (BLI) for monitoring bacteria-host interactions in farmed *H. midae* by i) developing a bioluminescent construct to label the probiotic bacterium *V. midae* SY9, ii) determining whether bioluminescent bacterial cells could be detected within the abalone digestive tract using this construct and iii) investigating whether this technique could be used to non-invasively monitor bacterial colonization of the abalone gastrointestinal tract.

Chapter 2

Materials and Methods

2.1 Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are listed in Table 2.1.

2.2 Media and culture conditions

2.2.1 Culturing *Escherichia coli*

E. coli strains were grown in Luria-Bertani broth (LB) (0.5% (w/v) NaCl, 1% (w/v), tryptone, 0.5% (w/v) yeast extract) and maintained on Luria-Bertani agar (LA) (LB supplemented with 1.5% (w/v) bacteriological agar) at 37°C. *E. coli* SM10λ*pir* harbouring pKluxCat (Table 2.1) was cultured in LB medium or maintained on LA solid medium supplemented with 15 $\mu g.ml^{-1}$ chloramphenicol (Sigma). *E. coli* JM109 harbouring plasmid pCAT19 (Table 2.1) was cultured in LB and maintained on LA solid medium supplemented with 30 $\mu g.ml^{-1}$ chloramphenicol. *E. coli* DH5α harbouring plasmid pAKlux2 was cultured in LB medium and maintained on LA solid medium supplemented with 100 $\mu g.ml^{-1}$ ampicillin (Sigma).

2.2.2 Culturing *Vibrio midae* SY9

Wild-type *V. midae* SY9 (Table 2.1) was cultured in tryptic soya broth (TSB) (1.7% (w/v) tryptone, 0.3% (w/v) peptone, 0.25% (w/v) glucose, 2.5% (w/v) di-potassium hydrogen phosphate, 3% (w/v) sodium chloride) and maintained on tryptic soya agar (TSA) (TSB supplemented with 1.5% (w/v) bacteriological agar). *V. midae* SY9 SR11 (Table 2.1 and section 2.4) was cultured in TSB medium supplemented with 120 $\mu g.ml^{-1}$ streptomycin (Sigma). *V. midae* SY9 K811 (Table 2.1) was cultured in TSB medium supplemented with 120 $\mu g.ml^{-1}$ streptomycin and 15 $\mu g.ml^{-1}$ chloramphenicol. All *V. midae* strains were incubated and grown at 30°C, unless otherwise stated.

Table 2.1 Bacterial strains and plasmids used in study.

Bacterial strain/plasmid	Genotypes/ relevant characteristic(s)[a]	Reference
Bacterial strains:		
Vibrio midae SY9	Isolated from the digestive tract of *Haliotis midae* abalone, South Africa	Macey (2005)
V. midae SY9 SR11	Sm^R strain of *V. midae* SY9	This study
V. midae SY9 K811	Sm^R and Cm^R strain of *V. midae* SY9, *luxCDABE*	This study
Escherichia coli SM10λ*pir*	*thi thr leu ton*A *lac*Y *sup*E (λ*pir*) *rec*A::RP4-2-Tc::Mu Km^R	Simon *et al.* (1983)
E. coli JM109	*recA*1 *supE*44 *endA*1 *hsdR*17 *gyrA*96 *relA*1 *thi*_(*lac-proAB*) F'(traD36 proAB' lacIq lacZ_M15)	Sambrook *et al.* (1989)
E. coli DH5α	dlacZ DeltaM15 Delta(lacZYA-argF) U169 *rec*A1 *end*A1 *hsd*R17(rK-mK+) supE44 thi-1 *gyr*A96 *re*lA1	Hanahan (1985)
Plasmids:		
pAKlux2	Amp^R, *lux*CDABE,	Karsi and Lawrence 2007
pCAT19	Derivative of pBR325 and pUC19 containing the 1053 bp Tn9-CAT cassette, Amp^R, Cm^R	Fuqua (1992)
pKluxCat	Derivative of pAKlux 2, containing the 1053bp BamHI-PstI Tn9-CAT fragment from pCAT19, Amp^R, Cm^R, *luxCDABE*	This study

[a]Sm^R, streptomycin resistant; Km^R, kanamycin resistant; Cm^R, chloramphenicol resistant; Amp^R, ampicillin resistant

2.3 Streptomycin resistant *Vibrio midae* SY9

A streptomycin resistant *V. midae* SY9 strain was selected for by spread-plating an overnight culture of the wild-type *V. midae* SY9 onto TSA/Sm gradient plates containing streptomycin ranging in concentration from 0–120 μg.ml^{-1}. Streptomycin gradient plates inoculated with *V*. *midae* SY9 were incubated at 22°C for approximately 24 hours. *V. midae* SY9 colonies growing at the higher streptomycin concentrations were repeatedly sub-cultured onto fresh TSA/Sm gradient plates until *V. midae* SY9 colonies capable of growth at 120μg.ml^{-1} were obtained. This strain was designated *V*. *midae* SY9 SR11.

2.4 Visualisation of bioluminescent cells

Bioluminescent imaging and measurements were performed with the Xenogen *in vivo* imaging system (IVIS) Lumina II (Caliper Life Science, MA, USA) (Fig. 2.1), and analysed using the Living Image software (version 3.2, Caliper Life Science, MA, USA) . The instrument allows real time imaging without sacrificing animals. The Xenogen IVIS Lumina II is able to detect bioluminescence and fluorescence at wavelengths in the range of 450-900 nm.

All bioluminescence data and images captured from the bioluminescent strains in this study were obtained by visualising both liquid and plate cultures of the strains in the Xenogen imaging system. The bioluminescent image data is measured and displayed in units of photons which is an absolute physical unit that measures photon emission from the object or organism of interest. The Living Image software allows pseudo-images to be generated where each pixel corresponds to a numerical value, which in turn is represented by a particular colour (Living image Software, user's guide). These pseudoimages are displayed in pseudocolours which represent the intensity of the bioluminescence measured. For this study, the rainbow colour scale was selected, with a red colour representing maximum bioluminescence intensity and a violet colour representing the minimum signal intensity. All bioluminescent data recorded was measured using photon flux, which represents the number of photons of light captured per second of exposure (phot.sec^{-1}).

The bioluminescence of bacterial plate cultures (both *V. midae* SY9 K811 and *E. coli* strains) viewed in the Xenogen was captured with an exposure time of 1 second, using an open filter and a field of view (FOV) of 10cm. The exposure time for the plate cultures was restricted to 1 second as longer exposure times led to oversaturated pixels. The liquid cultures of *V. midae* SY9 K811, were monitored for bioluminescence by imaging the cells in a black 96 well plate using a 1 minute exposure, an open filter and a FOV of 12.5cm. Animals inoculated with bioluminescent *V. midae* SY9 K811 were imaged in the Xenogen with an exposure time of 1 min, an open filter and field of view (FOV) of 10cm.

A)

B)

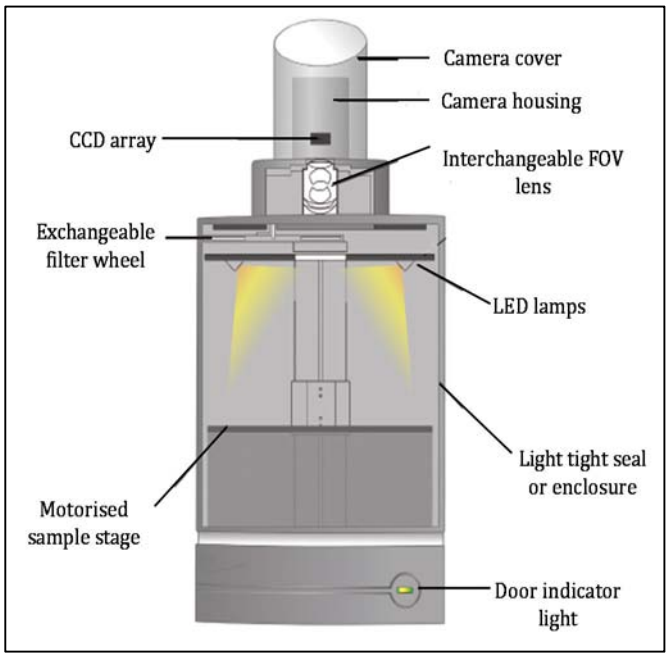

Figure 2. 1. The Xenogen *in vivo* imaging system (IVIS) Lumina II machine. A) The Xenogen machine used for bioluminescent detection. The image displays the outside view of the instrument. B) Schematic diagram of the inside of the Xenogen machine showing some of the important parts of the *in vivo* imaging system (Caliper Life Science, 2008).

2.5 Construction of plasmid pKluxCat containing the lux operon (luxCDABE) for expression of bioluminescence and the cat gene coding for chloramphenicol resistance

V. midae SY9 wt. was found to be highly sensitive to the antibiotic chloramphenicol and no growth of the bacterial strain was observed in media containing as little as 1 $\mu g.ml^{-1}$ chloramphenicol (data not shown). As a result, the chloramphenicol acetyltransferase (*cat*) gene, encoding chloramphenicol resistance, was cloned into the bioluminescent plasmid pAKlux2 (Table 2.1) to enable selection of *V. midae* SY9 trans-conjugants.

2.5.1 Plasmid DNA isolation

Plasmids pAKlux2 and pCAT19 were isolated from *E. coli* SM10λ*pir* and *E. coli* JM109, respectively, according to the method described by Ish-Horowicz and Burke (1981). Following the final centrifugation step of this method, the pellet containing plasmid DNA was re-suspended in a 50 µl tris-EDTA buffer (TE) (10mM Tris-Cl, 1 mM EDTA, pH 8). The isolated plasmid DNA was quantified using a Nanodrop spectrophotometer and stored at 4 °C until required.

2.5.2 Restriction enzyme digestion

Plasmids pCAT19 and pAKlux2 were digested with restriction enzymes *Bam*HI (Roche) and *Pst*I (Roche) according to the manufacturer's instructions. Each plasmid was subjected to a single and double restriction enzyme digest. Plasmids pCAT19 digested with *Bam*HI resulted in linearised plasmid DNA fragments of 2741 bp and 998 bp, while digestion with *Pst*I resulted in linearized plasmid DNA fragments of 2744 bp and 995 bp(Fig. 2.2). Digestion of plasmid pAKlux2 with *Bam*HI resulted in linear DNA fragments of 10800 bp and 25 bp in size, while digestion with *Pst*I yielded DNA fragments approximately 10789 bp and 27 bp in size (Fig. 2.3).

Plasmid pCAT19 subjected to a double digest with *Bam*HI and *Pst*I resulted in the excision of a 980 bp *cat* cassette (Fig 2.2). When plasmid pAKlux2 was subjected to a double digest with *Bam*HI and *Pst*I, a DNA fragment of roughly 43 bp was excised (Fig. 2.3), linearizing the plasmid. All restriction enzyme fragments were subjected to 1% agarose gel electrophoresis. The fragments of interest, a 980 bp cat gene fragment and the 10.782 kb pAKlux2 vector DNA fragment, were carefully excised and gel purified

using the BioSpin Gel Extraction Kit (BioFlux) according to the manufacturer's instructions.

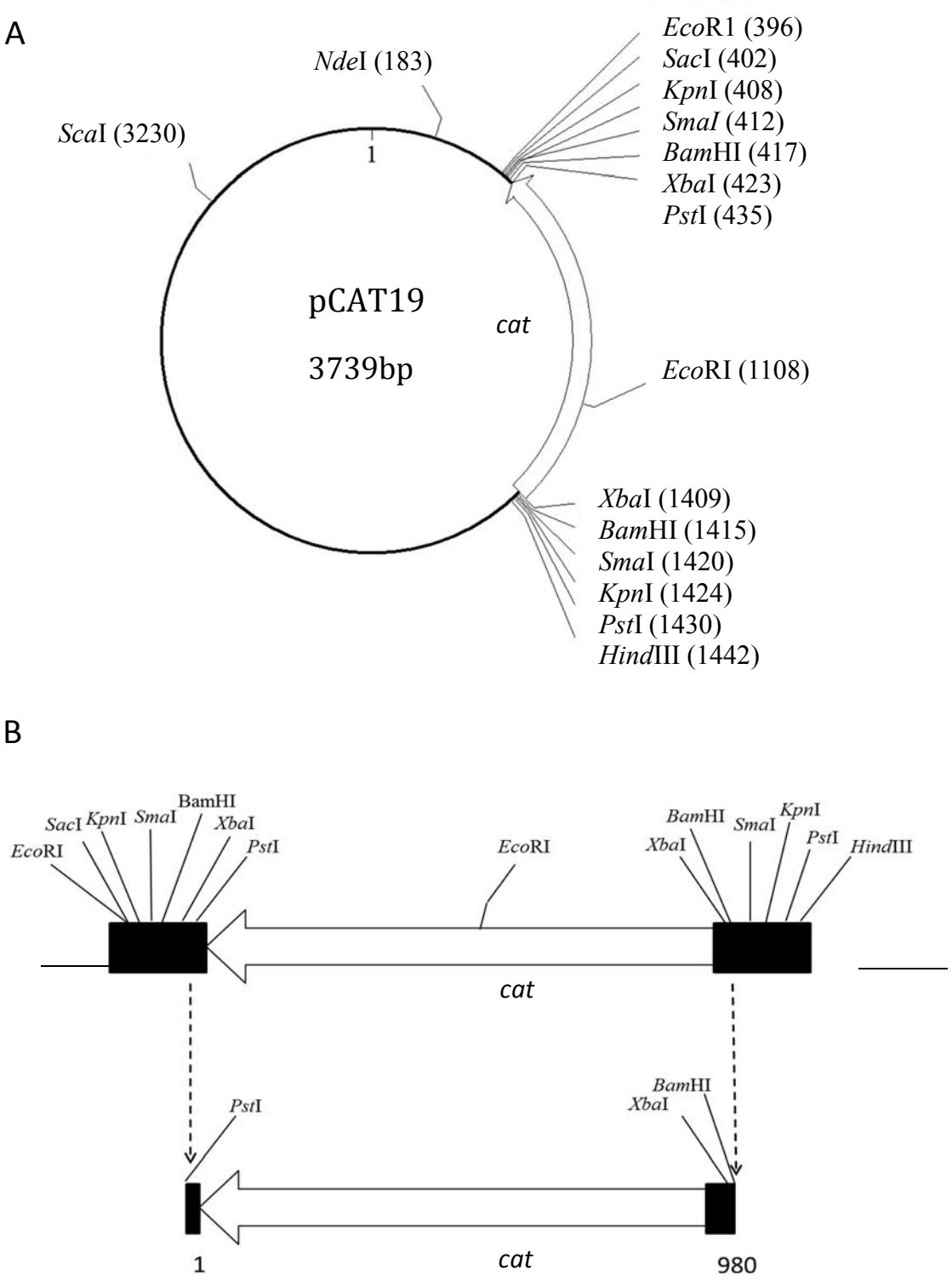

Figure 2. 2. Plasmid map of pCAT19 showing relevant restriction enzyme sites. A) Complete plasmid map. B) Excised DNA fragment (980bp) containing the chloramphenicol resistance (cat) gene.

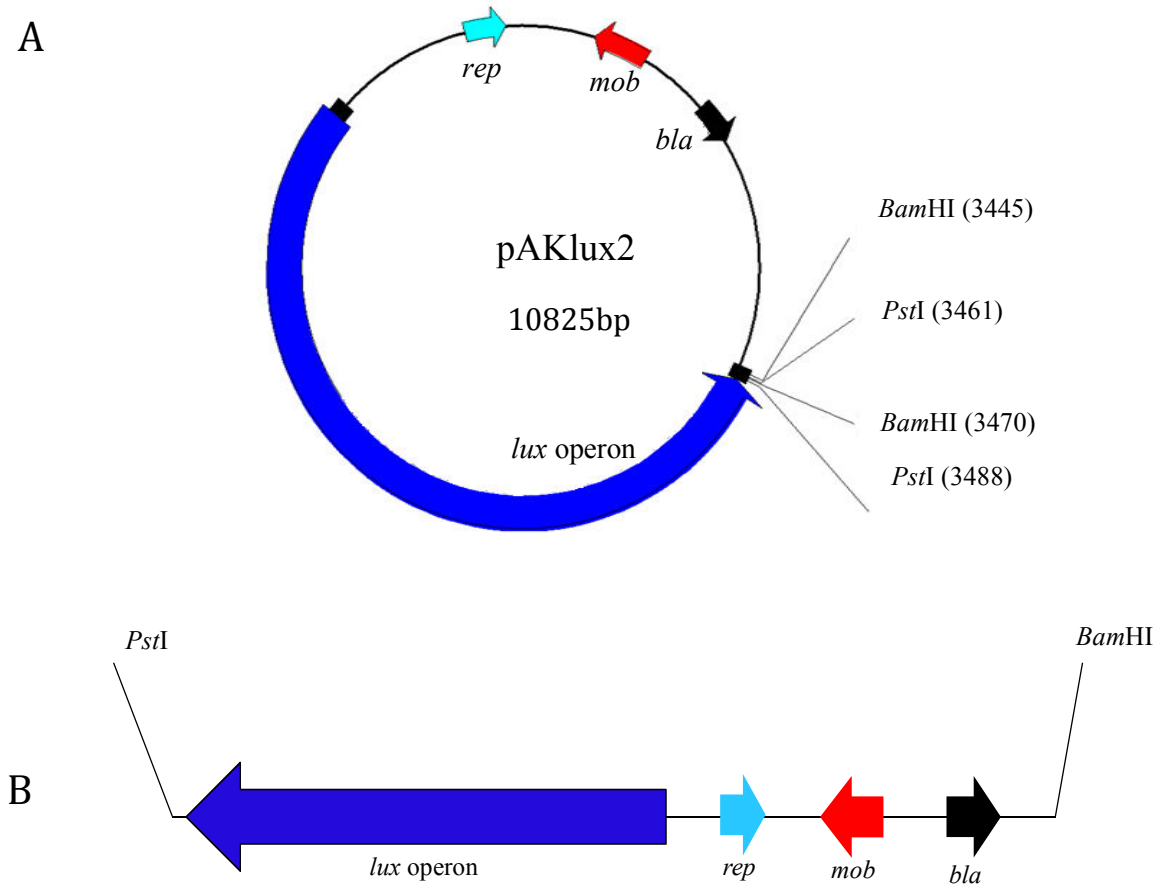

Figure 2. 3 Plasmid map of pAKlux2 showing relevant restriction enzyme recognition sites. A) Complete circularized plasmid map of pAKlux2 showing the positions of the *lux* operon ( ), rep gene ( ), *mob* gene ( ), *bla* gene ( ) and relevant restriction enzyme sites (Karsi & Lawrence, 2007). B) Linearized plasmid DNA after digestion with *Bam*HI and *Pst*I restriction enzymes.

2.5.3 Ligation and transformation

The 980 bp *Pst*I-*Bam*HI DNA fragment containing the full length cat gene excised from plasmid pCAT19 was cloned into pAKlux2 linearized with *BamH*I-*Pst*I. The vector to insert DNA (V: I) ratio was 1:4 and the total DNA concentration did not exceed 10pmol (Coyne *et al.,* 2002*;* Huddy, 2010). The reaction mixtures consisted of 2 µl of 10X T4 ligation buffer (Fermentas), 2 units of T4 DNA ligase and 100 ng of DNA made up to a total volume of 20 µl with sterile distilled water in 1.5 ml micro-centrifuge tubes. The reaction tubes were incubated at 22°C overnight. Plasmid pCAT19 and pAKlux2 DNA

which had been subjected to single restriction enzyme digests with either *Bam*HI or *Pst*I represented the controls. Additional controls included transformation of uncut vector DNA as well as *Bam*HI-*Pst*I double digested pAKlux2 DNA.

The resulting construct was transformed into competent *E. coli* SM10λ*pir* cells using the method described by Dagert and Ehrlich (1979). Following incubation, aliquots of cells were spread-plated on LA solid media supplemented with 30μg.ml^{-1} chloramphenicol and plates were incubated at 37 °C overnight to select for *E. coli* SM10λpir transformants. The cells transformed with single and double restriction enzyme digested vector DNA (pAKlux2) were plated on LA solid media supplemented with 100 μg.ml^{-1} ampicillin. Bacterial cells transformed with uncut pAKlux2 plasmid DNA were plated on media supplemented with 100 μg.ml^{-1} ampicillin and cells transformed with pCAT19 DNA were plated on LA solid media supplemented with 30 μg.ml^{-1} chloramphenicol. Following incubation, bioluminescent *E .coli* transformants were selected using the Xenogen (as described in section 2.4). The cells emitting a strong bioluminescent signal were then transferred and maintained on LA solid media supplemented with 30 μg.ml^{-1} chloramphenicol.

2.6 Confirmation of the insertion of the *cat* gene into plasmid pAKlux2

Plasmid pKluxCat DNA extracted using the method described in section 2.6.1 was sequenced using sequencing primers T3 (5'GCAATTAACCCTCACTAAAGG3') and $T7_{promoter}$ (TAATACGACTCACTATAGGG) (www.addgene.org). Sequence data was edited using Chromas version 1.45 (Connor McCarthy, 1994) and analysed using DNAMAN version 4.13 (Lynnon Biosoft). A protein homology search of the GenBank database was performed using the BLASTx program in NCBI.

2.7 PCR amplification of the chloramphenicol acetyltransferase *cat* gene.

Plasmid pCAT19 was isolated from *E. coli* JM109 as described in section 2.6.1. The purified plasmid DNA was subsequently used as template for amplification of the 824 bp *cat* gene by PCR. The Tn9 *cat* amplification forward primer Tn9F (5′-ATG AGA CGT TGA TCG GCA CG-3′) (T_m 53.7 °C) and reverse primer Tn9R (5′-ATG AGA CGT TGA TCG GCA CG-3′) (T_m 55.8 °C) were used in PCR reactions (Poteete *et al.*, 2006). Fig. 2.4 indicates where the primers bound to the cat DNA sequence. Plasmid pAKlux2 was used as a negative control. In addition, a non-template control (NTC; PCR-grade water) was analysed as a negative control to ensure there was no amplification except for the cat gene. The amplification reaction was initially optimized using a temperature gradient PCR (57.5 – 66.6 °C), with $MgCl_2$ concentrations ranging between 1 mM and 2.5 mM. The reaction mixtures contained 2.5 mM dNTPs, 0.5 U *Taq* polymerase and 4 µM each of Tn9F and Tn9R primers. Amplification was performed using a Bioer XP thermal cycler (Separation Scientific) and consisted of an initial denaturation at 95 °C for 300 s, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing (56-66.6 °C) for 30 s, and elongation at 72 °C for 45 s, followed with a final extension at 72 °C for 300 s. The amplified PCR product was analysed by 1 % agarose gel electrophoresis.

```
Tn9 CAT      GACGCACTTTGCGCCGAATAAATACCTGTGACGGAAGATC      40
Tn9F         ........................................       0
Tn9R.C prime........................................       0
Consensus
Tn9 CAT      ACTTCGCAGAATAAATAAATCCTGGTGTCCCTGTTGATAC      80
Tn9F         ........................................       0
Tn9R.C prime........................................       0
Consensus
Tn9 CAT      CGGGAAGCCCTGGGCCAACTTTTGGCGAAAATGAGACGTT     120
Tn9F         ..............................ATGAGACGTT      10
Tn9R.C prime........................................       0
Consensus                                    atgagacgtt
Tn9 CAT      GATCGGCACGTAAGAGGTTCCAACTTTCACCATAATGAAA     160
Tn9F         GATCGGCACG..............................      20
Tn9R.C prime........................................       0
Consensus    gatcggcacg
Tn9 CAT      TAAGATCACTACCGGGCGTATTTTTTGAGTTATCGAGATT     200
Tn9F         ........................................      20
Tn9R.C prime........................................       0
Consensus
Tn9 CAT      TTCAGGAGCTAAGGAAGCTAAAATGGAGAAAAAAATCACT     240
Tn9F         ........................................      20
Tn9R.C prime........................................       0
Consensus
Tn9 CAT      GGATATACCACCGTTGATATATCCCAATGGCATCGTAAAG     280
Tn9F         ........................................      20
Tn9R.C prime........................................       0
Consensus
Tn9 CAT      AACATTTTGAGGCATTTCAGTCAGTTGCTCAATGTACCTA     320
Tn9F         ........................................      20
Tn9R.C prime........................................       0
Consensus
Tn9 CAT      TAACCAGACCGTTCAGCTGGATATTACGGCCTTTTTAAAG     360
Tn9F         ........................................      20
Tn9R.C prime........................................       0
Consensus
Tn9 CAT      ACCGTAAAGAAAAATAAGCACAAGTTTTATCCGGCCTTTA     400
Tn9F         ........................................      20
Tn9R.C prime........................................       0
Consensus
Tn9 CAT      TTCACATTCTTGCCCGCCTGATGAATGCTCATCCGGAATT     440
Tn9F         ........................................      20
Tn9R.C prime........................................       0
Consensus
Tn9 CAT      CCGTATGGCAATGAAAGACGGTGAGCTGGTGATATGGGAT     480
Tn9F         ........................................      20
Tn9R.C prime........................................       0
Consensus
Tn9 CAT      AGTGTTCACCCTTGTTACACCGTTTTCCATGAGCAAACTG     520
Tn9F         ........................................      20
Tn9R.C prime........................................       0
Consensus
Tn9 CAT      AAACGTTTTCATCGCTCTGGAGTGAATACCACGACGATTT     560
Tn9F         ........................................      20
Tn9R.C prime........................................       0
Consensus
Tn9 CAT      CCGGCAGTTTCTACACATATATTCGCAAGATGTCGCGTGT     600
Tn9F         ........................................      20
Tn9R.C prime........................................       0
Consensus
Tn9 CAT      TACGGTGAAAACCTGGCCTATTTCCCTAAAGGGTTTATTG     640
Tn9F         ........................................      20
Tn9R.C prime........................................       0
Consensus
Tn9 CAT      AGAATATGTTTTTCGTCTCAGCCAATCCCTGGGTGAGTTT     680
Tn9F         ........................................      20
Tn9R.C prime........................................       0
Consensus
Tn9 CAT      CACCAGTTTTGATTTAAACGTGGCCAATATGGACAACTTC     720
Tn9F         ........................................      20
Tn9R.C prime........................................       0
Consensus
```

Continued...

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Tn9 CAT      TTCGCCCCCGTTTTCACCATGGGCAAATATTATACGCAAG     760
Tn9F         ........................................      20
Tn9R.C prime........................................       0
Consensus
Tn9 CAT      GCGACAAGGTGCTGATGCCGCTGGCGATTCAGGTTCATCA     800
Tn9F         ........................................      20
Tn9R.C prime........................................       0
Consensus
Tn9 CAT      TGCCGTCTGTGATGGCTTCCATGTCGGCAGAATGCTTAAT     840
Tn9F         ........................................      20
Tn9R.C prime........................................       0
Consensus
Tn9 CAT      GAATTACAACAGTACTGCGATGAGTGGCAGGGCGGGGCGT     880
Tn9F         ........................................      20
Tn9R.C prime........................................       0
Consensus
Tn9 CAT      AATTTTTTTAAGGCAGTTATTGGTGCCCTTAAACGCCTGG     920
Tn9F         ........................................      20
Tn9R.C prime.....................................GCCTGG      6
Consensus                                        gcctgg
Tn9 CAT      TGCTACGCCTGAATAAGTGATAATAAGCGGATGAATGGCA     960
Tn9F         ........................................      20
Tn9R.C primeTGCTACGCCTGAAT.........................      20
Consensus    tgctacgcctgaat
Tn9 CAT      GAAATTCGAAAGCAAATTCGACCCGGTCGTCGGTTCAGGG    1000
Tn9F         ........................................      20
Tn9R.C prime........................................      20
Consensus
Tn9 CAT      CAGGGTCGTTAAATAGCCGCTTATGTCTATTGCTGGTTTA    1040
Tn9F         ........................................      20
Tn9R.C prime........................................      20
Consensus
Tn9 CAT      CCGGTTTATTGACTACCGGAAGCAGTGTGACCGTGTGCTT    1080
Tn9F         ........................................      20
Tn9R.C prime........................................      20
Consensus
Tn9 CAT      CTCAAATGCCTGAGGCCAGT                        1100
Tn9F         ....................                          20
Tn9R.C prime....................                          20
Consensus
```

Figure 2. 4: Nucleotide sequence alignment of the of Tn9F and Tn9 reverse *cat* gene primers. The highlighted nucleotide sequences (blue) indicates the binding position of the primers on the *cat* gene nucleotide sequence.

2.8 Screening of *E. coli* SM10λ*pir* transformants for the presence of plasmid pKluxCat

E. coli SM10λpir cells were streak inoculated onto LA media supplemented with 30 μg.ml^{-1} chloramphenicol and incubated at 37 °C overnight. The plate culture was viewed in the Xenogen to evaluate the presence of a bioluminescent signal as described (2.4). In addition, a single bacterial colony was picked and suspended in 25 μl of sterile water in a sterile 1.5 ml micro-centrifuge tube. The tube was incubated at 95°C for 15 – 20

minutes to lyse the cells before being centrifuged at 5000 x g for 10 minutes at room temperature. Four microliters of the resulting supernatant, containing both plasmid and genomic DNA, was removed and served as the template for the subsequent PCR reaction as described (2.8). The following cycling conditions were used: 95 °C for 300 s, 35 cycles of 95 °C for 30 s, 66.6 °C for 30 s, 72 °C for 45 s, followed by elongation at 72 °C for 300 s. The PCR products were analysed on a 1% TAE agarose gel (containing ethidium bromide), and imaged using the Chemidoc system (BIORAD) and Image lab software (BIORAD, version 2.0.1).

2.9 Labelling of *Vibrio midae* SY9 with bioluminescent plasmid pKluxCat

2.9.1 *Vibrio midae* SY9 conjugation

Briefly, *E. coli* SM10λ*pir* (donor) was cultured overnight in 5 ml LB supplemented with 15 μg.ml^{-1} chloramphenicol at 37 °C. *V. midae* SY9 SR11 (recipient) was cultured in peptone broth (2.5% (w/v) sodium chloride and 1.5% (w/v) peptone), supplemented with 120 μg.ml^{-1} streptomycin at 22 °C for 24 hours. Approximately 500 μl of the donor and 1500 μl of the recipient strain were placed into 1.5 ml micro-centrifuge tubes. The cells were harvested by centrifugation (5000 x g for 5 minutes at 22 °C) and washed twice with 500 μl of fresh peptone broth before resuspending the bacterial pellets in 50 μl peptone broth. Equal volumes (1:1) of resuspended donor and recipient cells were combined and gently mixed before the cells were concentrated by centrifugation. The resulting bacterial pellet, or mating mix, was resuspended in 50 μl peptone broth and spot inoculated onto 0.45 μm HAWP filters (Millipore) that had been placed on the surface of TSA agar. Fifty microliters of the donor and recipient cells were also separately inoculated onto a membrane that had been placed on the surface of TSA solid media. The plates were incubated at 28 °C for 18 hours, after which the cells were recovered by placing the membrane filter into a sterile 10 ml centrifuge tube containing 500 μl of peptone broth. The tubes were vortexed to release the bacteria, before removing the membrane and centrifuging at 6500 x g for 5 minutes to concentrate the cells. The resultant bacterial pellet was re-suspended in 500 μl peptone broth supplemented with 120 μg.ml^{-1} streptomycin and 15 μg.ml^{-1} chloramphenicol.

One hundred microliter aliquots of the re-suspended cells were either spot inoculated onto TSA plates supplemented with 120 μg.ml^{-1} streptomycin and 15 μg.ml^{-1} chloramphenicol, or serially diluted and then inoculated by spread plate technique onto this medium. The plate cultures were incubated at 15 °C for three days, after which potential trans-conjugants were selected by checking for bioluminescence using the Xenogen IVIS Lumina II according to the methods described in section 2.4 above. Colonies that displayed bioluminescent activity were sub-cultured onto thiosulfate-citrate-bile salts-sucrose agar (TCBS, Merk) to confirm that the selected bacteria were *Vibrio*.

2.9.2 Marker stability

V. midae SY9 K811 trans-conjugants harbouring pKluxCat were repeatedly sub-cultured on TSA solid media that either lacked antibiotics or were supplemented with 15 μg.ml^{-1} chloramphenicol and 120 μg.ml^{-1} streptomycin to determine the stability of the plasmid. The bioluminescent activity of bacterial cells growing on solid media with and without antibiotics was also monitored. A 5 ml overnight culture of *V. midae* SY9 K811 in TSB supplemented with 120 μg.ml^{-1} streptomycin and 15 μg.ml^{-1} chloramphenicol was used to inoculate 50 ml TSB supplemented with the same concentration of antibiotics. The culture was incubated at 30 °C for approximately 8 hours with shaking at 100 rpm. An aliquot of the culture was then removed and ten-fold serial dilutions were performed. One hundred microliter aliquots of the serially diluted culture were spread-plated onto TSA supplemented with antibiotics. The plates were incubated at 30°C for 24 hours, after which they were viewed in the Xenogen to check for bioluminescent colonies (2.4). One hundred randomly selected bioluminescent colonies were spot-inoculated onto TSA agar plates supplemented with 120 μg.ml^{-1} streptomycin and 15 μg.ml^{-1} chloramphenicol. Plates were incubated at 30°C for 24 hours, after which colonies were transferred onto TSA plates with and without antibiotics. The plates were incubated at 30 °C for 24 hours. The number of colonies retaining the plasmid was calculated by comparing the ability of randomly selected colonies, grown without antibiotic selection, to subsequently grow in the presence and absence of streptomycin and chloramphenicol. The colonies were also viewed in the

Xenogen to monitor how long they retained their ability to bioluminescence in the absence and presence of the antibiotics.

2.9.3 PCR amplification of Tn9 cat gene from *V. midae* SY9 K811 trans-conjugant cells.

To further verify that the *V. midae* SY9 K811 trans-conjugants growing on media supplemented with both streptomycin and chloramphenicol had acquired plasmid pKluxCat, a PCR amplification was performed using Tn9 *cat* amplification primers. Plasmid DNA was isolated from strain SY9 K811 transconjugants using the colony PCR method and cycle conditions described in section 2.7 above. PCR products were analysed by 1 % agarose gel electrophoresis to verify reaction specificity and product size (824 bp)

2.9.4 Determination of the bacterial growth profiles of *V. midae* SY9 wt. and *V. midae* SY9 K811.

Bacterial growth curve analysis of *V. midae* SY9 wt. and *V. midae* SY9 K811 was performed to compare the growth profiles of the two strains in order to investigate whether pKluxCat had any effect on the growth of *V. midae* SY9 transconjugants. Simultaneously, this experiment was performed in order to determine at which phase of growth bioluminescence was maximally emitted by the labelled bacterial cells.

V. midae SY9 wt. was cultured in 5 ml of TSB media on an orbital shaker at 30 °C overnight. Strain SY9 K811 was used to inoculate 5 ml of TSB media with and without antibiotic supplementation (120 $\mu g.ml^{-1}$ streptomycin and 15 $\mu g.ml^{-1}$ chloramphenicol), and the cultures were shake flask cultivated overnight at 30 °C. Following incubation, the 5 ml cultures of *V. midae* SY9 K811 and wild-type SY9 (initial absorbance of 0.05) were separately inoculated into 1L flasks (in triplicate) containing 100 ml of TSB. Half of the flasks were supplemented with antibiotic. Thus, for each strain three flasks contained TSB and antibiotic while three contained TSB alone. The cultures were incubated at 30 °C with shaking at 100 rpm. Triplicate samples (900 µl) were removed from each flask at 1 hour intervals for 24 hours after which the optical density at

600 nm was measured using a spectrophotometer (Novaspec). Subsequently, the cells removed from the flasks were concentrated by centrifugation at 5000 x g for 5 minutes at room temperature. The cells were washed once in TSB, and resuspended in 150 µl of fresh TSB media. Sample bioluminescence was then measured in triplicate (50 µL aliquots) in black 96 well microtitre plates, using the Xenogen as described (2.4).

2.9.4.1 Investigation of the observed decrease in *V. midae* SY9 K811 bioluminescence cultured in the absence of antibiotics.

The bacterial growth curve of *V. midae* SY9 K811, cultured in the absence of antibiotics, was repeated to determine whether the observed decrease in bioluminescence was as a result of bacterial death or plasmid loss. One hundred microliter aliquots of each culture were removed after 0, 3, 6, 16, 20 and 24 hours and a 10 fold serial dilution was performed. Aliquots of the serial dilutions were then spread plated onto TSA media with and without streptomycin and chloramphenicol. The inoculated plates were incubated at 30 °C overnight, after which they were placed in the Xenogen to select for bioluminescent colonies. Plate cultures (cultures present in the wells of a 96 well plate) were imaged with an exposure time between 1 - 10 seconds and FOV of 10 cm. The total bacterial number (cfu.ml^{-1}) was enumerated at each time point by colony counts. The presence of plasmid pKluxCat in bioluminescent colonies was confirmed by PCR amplification using the Tn9 *cat* gene primers and PCR conditions described in section 2.8.

2.10 Lower detection limit of *V. midae* SY9 K811 bioluminescence.

Three single *V. midae* SY9 K811 colonies were inoculated separately into 5 ml TSB media supplemented with 120 µg.ml^{-1} streptomycin and 15 µg.ml^{-1} chloramphenicol. The 5 ml cultures were incubated at 30 °C overnight, and subsequently used to inoculate three 1000 ml flasks containing 100 ml TSB media. The 100ml cultures were then incubated, with shaking, at 30 °C for 5 hours, until the cultures were in the mid-log phase of growth. Thereafter, a 1 ml aliquot was removed from each of the three flasks and transferred to three sterile 1.5 ml microcentrifuge tubes. The cells were then

pelleted by centrifugation at 5000 x g for 5 minutes at room temperature. Following centrifugation, the pellets were washed once (5 minutes at 5000 x g) and resuspended in 200 µl TSB media. A 2-fold serial dilution (15 dilutions) was performed and 100 µl aliquots of the dilutions were transferred to a 96 well microtitre plate and bioluminescence was measured as described (section 2.5.) Following imaging, the bacterial suspension was removed, spread plated onto TSA plates supplemented with 120 $\mu g.ml^{-1}$ streptomycin and 15 $\mu g.ml^{-1}$ chloramphenicol, and incubated at 30 °C for 24 hours. Thereafter, the number of colony forming units (cfu) was determined.

2.11 Detection of bioluminescent *V. midae* SY9 K811 *in vivo*

2.11.1 Animals

Juvenile *H. midae* abalone (4.2 ± 0.07 mm shell length; n=24) were maintained in two 60 L glass aquaria (12 animals per tank) housed in a temperature controlled laboratory maintained at 18 °C at the University of Cape Town. Unfiltered air was supplied to tanks containing 40 L of 10 µm filtered natural sea water. The sea water was allowed to recirculate and filtration was achieved through an external canister filter equipped with activated charcoal and a membrane filter. Abalone were maintained in the dark, to encourage feeding, and were only exposed to light when the tanks were cleaned or when animals were fed. Abalone were fed fresh kelp every second day and the tanks were cleaned twice a week, by siphoning, to remove any uneaten feed and faeces. The water was completely replaced once or twice a week, depending on the condition of the water) by removing all the water and replacing it with 40L fresh filtered sea water (acclimatised to 18 °C).

2.11.2 *In vivo* detection of bioluminescent *V. midae* SY9 K811.

V. midae SY9 K811 was cultured overnight in 5 ml of TSB media supplemented with 120 $\mu g.ml^{-1}$ streptomycin and 15 $\mu g.ml^{-1}$ chloramphenicol. The overnight culture was used to inoculate 100 ml of TSB media to an initial absorbance of 0.05 at 600 nm. The culture was incubated at 30°C with shaking at 100 rpm for 5 hours. One millilitre aliquots of the culture were dispensed into 12 separate sterile 1.5 ml microcentrifuge tubes and cells

were concentrated by centrifugation at 5000 x g for 5 minutes. The supernatant was removed and the bacterial pellets were resuspended in 5 µl of fresh TSB media.

The bacterial suspensions, at a concentration of 7.8 x 10^7 cfu.ml^{-1}, were introduced orally (oral gavage) into 12 juvenile abalone that had been anesthetised by immersion in seawater supplemented with 5% (w/v) magnesium chloride for 5 - 10 minutes. The animals were placed in the Xenogen immediately after administration of the bacterial inoculum to determine bioluminescence (T = 0) and to determine the extent, if any, of spillage from the site of inoculation. The bioluminescent activity was evaluated using the living image software (Version 3.2.) by imaging animals with an exposure time of 60 s, using an open filter and a FOV of 12.5 cm. Images of the abalone were taken before and after the foot muscle was gently wiped with moist tissue paper to remove excess mucous and attached particles. This also ensured removal of any bacterial cells which could have leaked out of the inoculation site during oral administration.

The animals were immediately returned to the holding tank after imaging. Additional bioluminescence measurements were recorded 1, 2, 4, 6, 8, 10 and 12 hours post-inoculation to determine how long the bacterial cells remained metabolically active within gut of the abalone by monitoring the bioluminescent signal. Animals were anesthetised by immersion in 5% magnesium sulphate for 5-10 minutes before each imaging point.

2.12 Statistical analysis

All data are presented as the means of at least three values and standard deviation or standard errors. One factor analysis of variance (ANOVA), followed by the multiple comparison test (Tukey), was used to determine significant differences for bioluminescent measurements obtained from animals fed bioluminescent *V. midae* SY9.

Chapter 3

Results

3.1 Construction of plasmid pKluxCat containing the lux operon (luxCDABE) for expression of bioluminescence and chloramphenicol acetyltransferase *(cat)* gene coding for chloramphenicol resistance.

Plasmid pAKlux2, which contains the bacterial *lux* operon (bioluminescence expression) and the beta-lactamase (*bla*) gene coding for ampicillin resistance, was constructed with the aim to label a broad range of Gram-negative bacteria. *V. midae* SY9 cells transformed with plasmid pAKlux2 by conjugal mating were however found to be highly resistant to ampicillin. In contrast, *V. midae* SY9 was found to be highly sensitive to chloramphenicol (data not shown). Thus, the chloramphenicol acetyltransferase (*cat*) gene from plasmid pCAT19 was excised and cloned into plasmid pAKlux2, resulting in a new plasmid construct referred to as pKluxCat to allow for better counter-selection of *V. midae* SY9 trans-conjugants.

Plasmids pAKlux2 and pCAT19 were both digested with the restriction endonucleases *Bam*HI and *Pst*I. Digestion of plasmid pCAT19 DNA with the restriction endonucleases *Bam*HI and *Pst*I yielded two DNA fragments of approximately 2.726 kb and 980 bp in size, with the latter fragment containing the *cat* cassette (Fig. 3.1, lanes 4-6). Digestion of plasmid pAKlux2 with restriction enzymes *Bam*HI and *Pst*I resulted in the visualisation of only 1 DNA band of 10.782 whereas the expected 43 bp restriction fragment was not visible on the gel due to its small size (Fig. 3.1, lanes 10 - 12).

The 980 bp restriction fragment from pCAT19, containing the intact *cat* cassette, was ligated to the 10.782 kb restriction fragment from the linearized vector pAKlux2 and the resulting ligation mix was transformed into *E. coli* SM10λ*pir* cells. The *E. coli* SM10λ*pir* cells, which were previously chloramphenicol sensitive, were able to grow on media supplemented with 15 $\mu g.ml^{-1}$ chloramphenicol. Xenogen imaging of the transformants clearly demonstrated the ability of the *E. coli* cells to bioluminescence (Fig. 3.2), confirming the introduction and expression of pKluxCat (Chapter 2, Table 2.1) in the *E. coli* SM10λp*ir* cells.

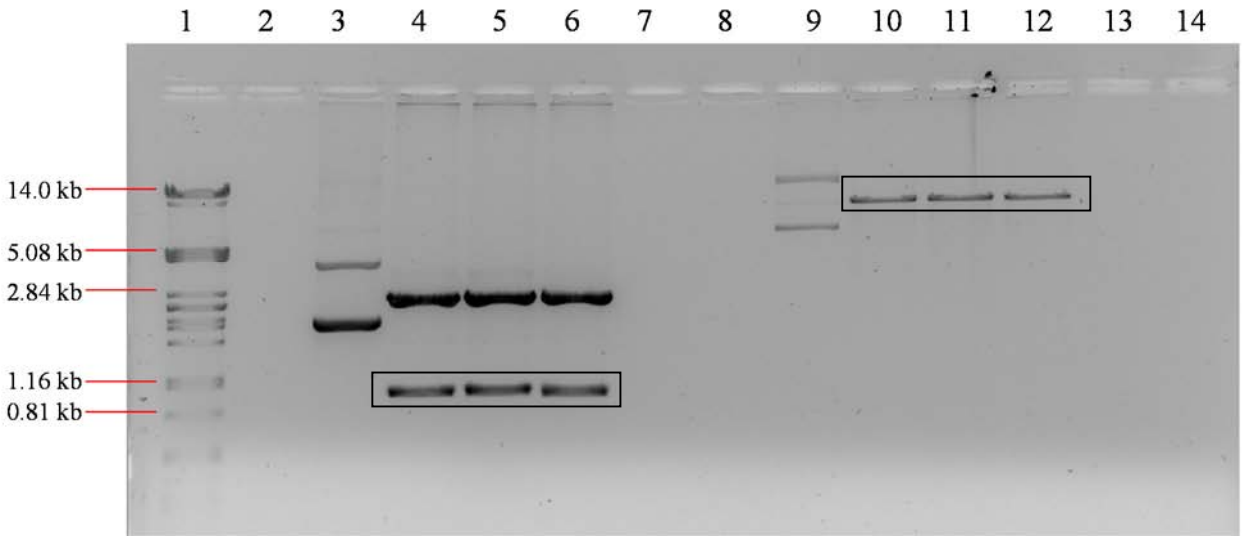


Figure 3. 1. Analysis of DNA fragments after restriction enzyme digestion. DNA fragments were separated on a 1% TAE agarose gel containing ethidium bromide (10 µg.ml^{-1}). Lanes 1) λ-*Pst* DNA marker, 2) empty, 3) uncut pCAT19 DNA, 4) pCAT19 digested with *Bam*HI, 5) pCAT19 digested with *Pst*I and 6) pCAT19 digested with *Bam*HI and *Pst*I, 7-8) empty, 9) Uncut pAKlux2 DNA, 10) pAKlux2 digested with *Bam*H1, 11) pAKlux2 digested with *Pst*I and 12) pAKlux2 digested with *Bam*HI and *Pst*I. The boxes around the restriction fragments indicate the DNA fragments that were excised from the gel. Lanes 4-6) show the *cat* gene cassette and lanes 10-12) shows the linearised pAKlux2 vector DNA.

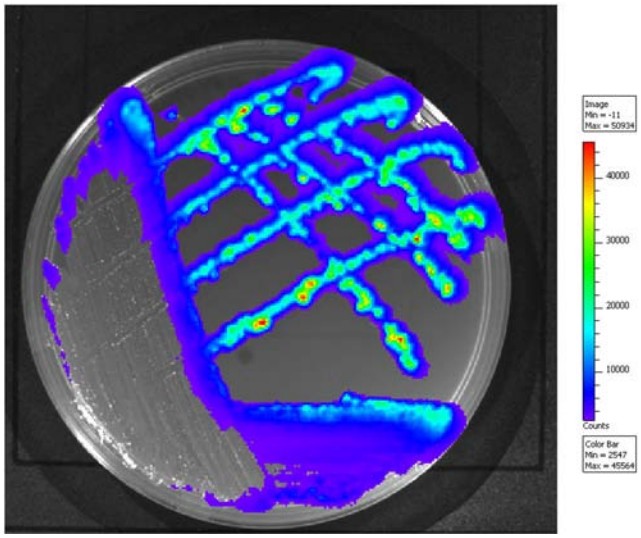


Figure 3. 2. Plate culture of *E. coli* SM10λ*pir* cells harbouring plasmid pKluxCat. The pseudocolour overlay, as quantified using the living image software, is an indication of the bioluminescence produced by these bacterial cells.

To confirm that the *cat* gene had been correctly cloned into pAKlux2, plasmid pKluxCat DNA was isolated from a positive *E. coli* transformant and sequenced using the T7 and T3 primers. BLASTx analysis of edited sequences confirmed the presence of the *cat* gene (Table 3.1) downstream from the T7 primer binding site and the *lux*C gene downstream from the T3 primer (Fig. 3.3), thus indicating that the cat gene had been cloned on the 3' end of the *lux* operon.

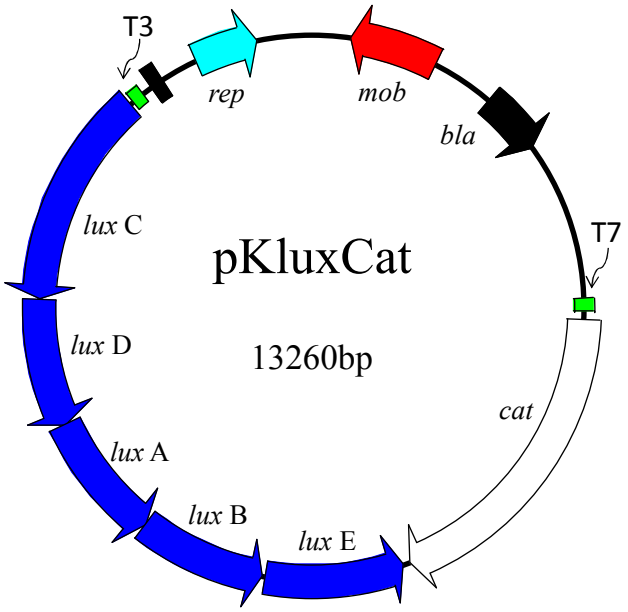


Figure 3. 3. Plasmid map of the construct pKluxCat formed by ligating the chloramphenicol resistance gene (cat) into plasmid pAKlux2. The blue arrows ( ) represent the *lux* genes (*lux*CDABE) that make up the lux operon, the black arrow ( ) represents the *bla* gene coding for ampicillin resistance and the clear arrow ( ) represents the *cat* gene coding for chloramphenicol resistance.

Table 3. 1. List of protein identities showing homology to the Tn9 chloramphenicol acetyltransferase gene sequenced from plasmid pKluxCat using the T7 promoter sequencing primer.

Description	% identity	E-value	Query coverage	Accession number
Chloramphenicol acetyltransferase, partial (*Salmonella enterica* subsp.)	98%	7×10^{-85}	95%	AEQ98813.1
Chloramphenicol acetyltransferase, partial (*Acinetobacter bauaman*)	83%	8×10^{-85}	99%	AEQ37464.1
Chloramphenicol acetyltransferase (unidentified cloning vector)	98%	4×10^{-84}	99%	AAA57080.1
Cm^R MISSA donor vector	98%	5×10^{-84}	99%	ADC79570.1
Chloramphenicol acetyltransferase, (*Enterococcus faecalis*)	98%	6×10^{-84}	99%	WP002361567.1

3.2 PCR amplification of the Tn9 *cat* gene

Polymerase chain reaction (PCR) is a standard molecular technique that has been used in many applications for rapid screening of an insert of interest (gene on a plasmid) for example. The ability to design primers specific to that insert of interest, allows for accurate and rapid identification of the DNA element within a host organism. A PCR protocol was optimised to efficiently screen for plasmid pKluxCat within *V. midae* SY9 trans-conjugants.

3.2.1 PCR optimisation

PCR optimisation for amplification of the Tn9 *cat* gene with the Tn9F/R primer set was conducted by varying the magnesium chloride concentration in the PCR reaction mix and using a temperature gradient protocol to optimise the annealing temperature. Plasmid pCAT19 DNA was used as the DNA template for PCR optimisation.

A DNA fragment of the expected size (824 bp) was amplified at a magnesium chloride concentration of 1.5 mM (Fig. 3.4, Lane 5), and it was noted that magnesium chloride

concentrations lower than this prevented amplification of the *cat* gene (Fig. 3.4, Lanes 3 & 4). Although other DNA bands were present on the agarose gel, the expected 824 bp *cat* gene fragment was not present for magnesium concentrations lower than 1.5mM. Similarly, non-specific DNA bands were also observed at a magnesium chloride concentration of 2.5 mM for PCR reactions containing plasmid pAKlux2 (negative control) (Fig. 3.4, Lane 11). Some non-specific DNA fragments were amplified off of the pCAT19 plasmid DNA template (Fig.3.4, Lanes 3-6). The bands however corresponded to the DNA fragments amplified in the PCR reactions containing the forward only and reverse only Tn9 cat primers (Fig. 3.5, Lanes 13 and 14). It was for this reason that further PCR optimisation was conducted and this included optimising the annealing temperature for the PCR reaction.

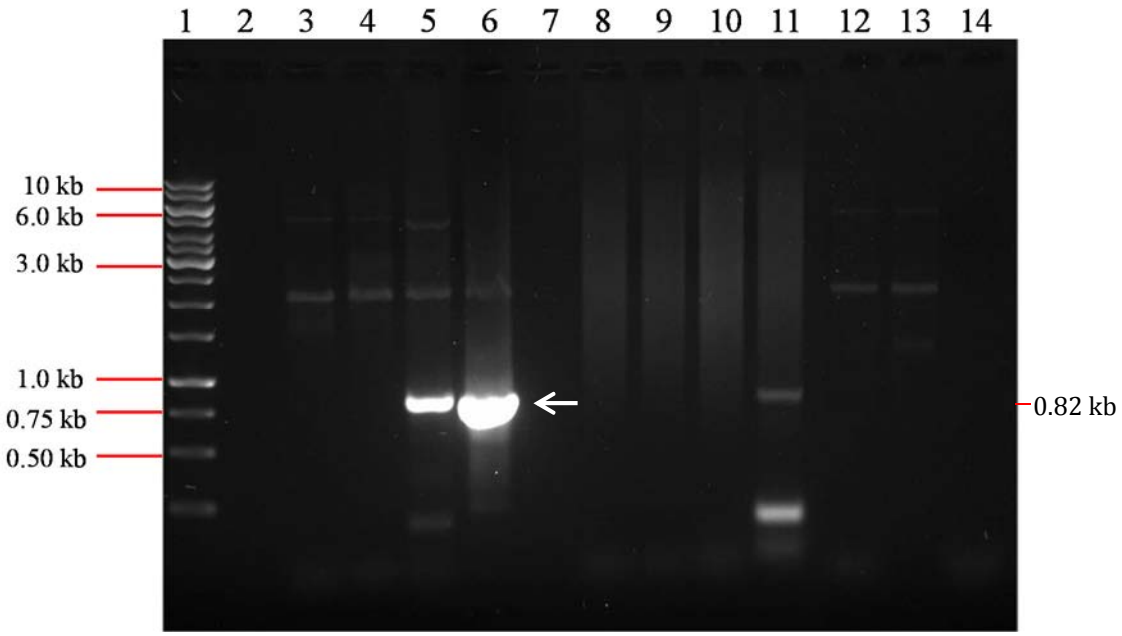


Figure 3. 4. Separation of PCR products amplified using various magnesium chloride concentrations in the PCR reactions. Lanes 1) 1kb DNA ladder, 2) empty, 3) 1 mM $MgCl_2$, 4) 1.5 mM $MgCl_2$, 5) 2 mM $MgCl_2$, 6) 2.5 mM $MgCl_2$. Lanes 3-6 represent PCR reactions amplified with plasmid pCAT19 as a DNA template. Lanes 8) 1 mM $MgCl_2$, 9) 1.5 mM $MgCl_2$, 10) 2 mM $MgCl_2$, 11) 2.5 mM $MgCl_2$. Lanes 8-11 represent PCR reactions amplified with plasmid pAKlux2 as a DNA template. Lanes 12) Forward primer only control, 13) Reverse primer only control, 14) No template control (NTC). The black white arrow indicates a DNA fragment that corresponds to the size of the *cat* gene.

It was further demonstrated that an annealing temperature of 66.6 °C was optimal for amplification of the *cat* gene because it was only at this temperature that no PCR product was detected for plasmid pAKlux2 (negative control) when the PCR reaction was subjected to gel electrophoresis (Fig. 3.5, Lane 15). Once again some non-specific DNA bands were present for PCR reactions using plasmid pAKlux2 as a DNA template; however, the presence of the *cat* gene fragment was the main focus.

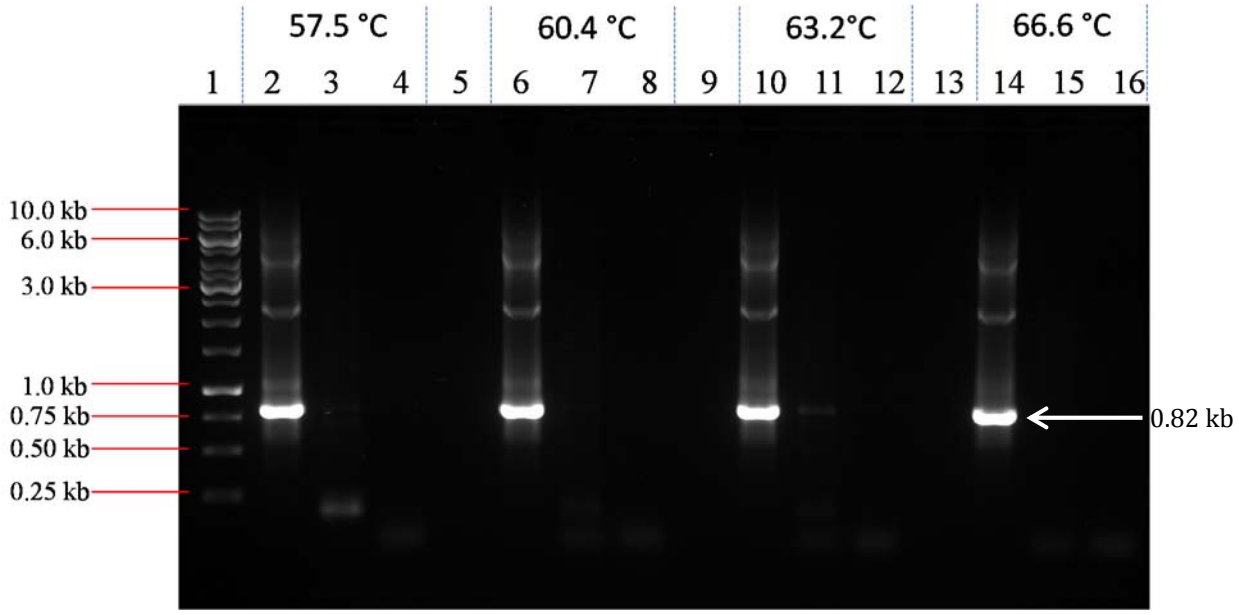


Figure 3. 5. Separation of PCR products amplified using temperature gradient PCR for optimising the annealing temperature. Lanes 1) 1Kb DNA ladder; Lanes 2, 6, 10 and 14) PCR product using pCAT19 as template (positive control); Lanes 3, 7, 11 and 15) PCR product using pAKlux2as template (negative); Lanes 4, 8, 12 and 16) No template controls (NTC). The white arrow on the right indicates PCR amplification products, that corresponds to the size of the *cat* gene (~ 0.82 kb).

The optimised PCR conditions were used to screen for the presence of plasmid pKluxCat in transformed *E. coli* SM10λ*pir* cells. Agarose gel electrophoresis of PCR products resulted in the visualisation of an 824 bp DNA band (Fig. 3.6, Lane 3). A DNA fragment of identical size was also visualised following PCR amplification with plasmid pCAT19 DNA as template (positive control) (Fig. 3.6, lane 2). This confirmed the presence of plasmid pKluxCat within the *E. coli* transformant, and demonstrated that the Tn9 *cat* primers could be used to screen for cells harbouring this plasmid.

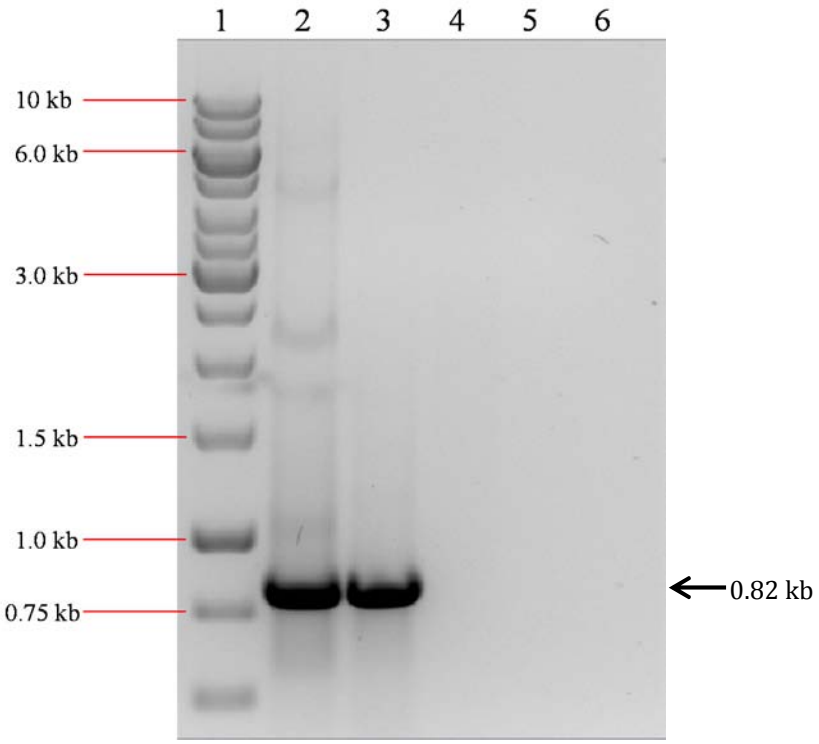


Figure 3. 6. Colony PCR screening of *E. coli* transformant cell. Lanes 1) 1Kb DNA ladder, 2) pCAT19 plasmid DNA (positive), 3) E. coli SM10λ*pir* transformant (colony), 4) pAKlux2 plasmid DNA (negative), 5) empty, 6) No template control (NTC). The black arrow on the right of the image indicates the expected PCR amplification product which correspond to the size of the cat gene (~0.8 kb)

3.3 Labelling of *V. midae* SY9 SR11 with bioluminescent plasmid pKluxCat

3.3.1 Conjugal transfer of plasmid pKluxCat into *V. midae* SY9 K811

Conjugal transfer of plasmid pKluxCat from *E. coli* SM10λ*pir* to *V. midae* SY9 SR11 (Chapter 2, Table 2.1) occurred at a high frequency (300 cfu.ml^{-1} of undiluted conjugal mix) however, only a few of the colonies were positive for bioluminescence when viewed in the Xenogen. Seven colonies that were strongly bioluminescent were selected for further analysis (Fig. 3.7). Subsequent transfer of colonies onto TCBS agar revealed that only three out of the seven colonies grew, indicating that they were *Vibrio* bacteria.

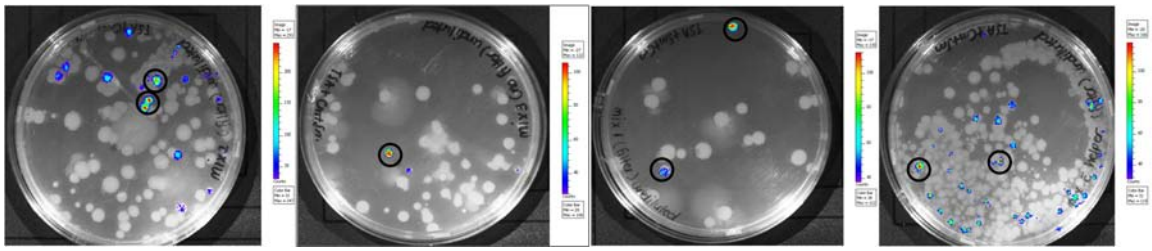


Figure 3. 7. *V. midae* SY9 trans-conjugants harbouring plasmid pKluxCat. The colonies circled in black had the highest bioluminescent signal, and were selected for further analysis. The selected colonies were able to grow on both the TSA and TCBS selective media, indicating that the trans-conjugants are *Vibrio* strains that are resistant to streptomycin and chloramphenicol.

The colonies were re-streaked onto TSA media supplemented with chloramphenicol and streptomycin and plate cultures were viewed in the Xenogen following 24 hours of growth. The three bioluminescent cultures were designated *V. midae* SY9 K811 Col 1, Col 12 and Col 16 (Fig. 3.8).

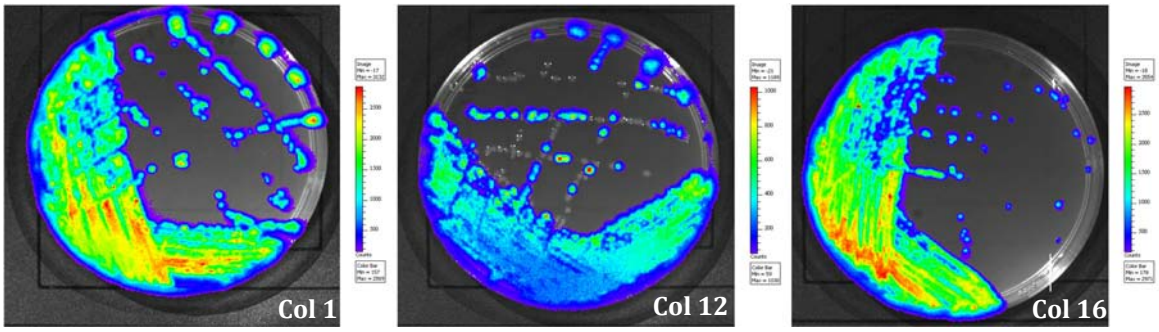


Figure 3. 8. Xenogen images of *V. midae* SY9 trans-conjugant colonies 1, 12 and 16: harbouring plasmid pKluxCat.

V. midae SY9 K811 Col 1, Col 12 and Col 16 were subjected to PCR amplification using the Tn9 *cat* gene specific primers (Tn9F and TN9R) to further confirm the presence of plasmid pKluxCat. Analysis of the PCR products revealed the presence of a single band approximately 824 bp in size (Fig. 3.9, Lanes 3 - 4). The band size corresponded to the DNA band amplified for the positive control (Fig. 3.9, lane 2), thereby confirming the presence of the cat gene, and in turn plasmid pKluxCat.

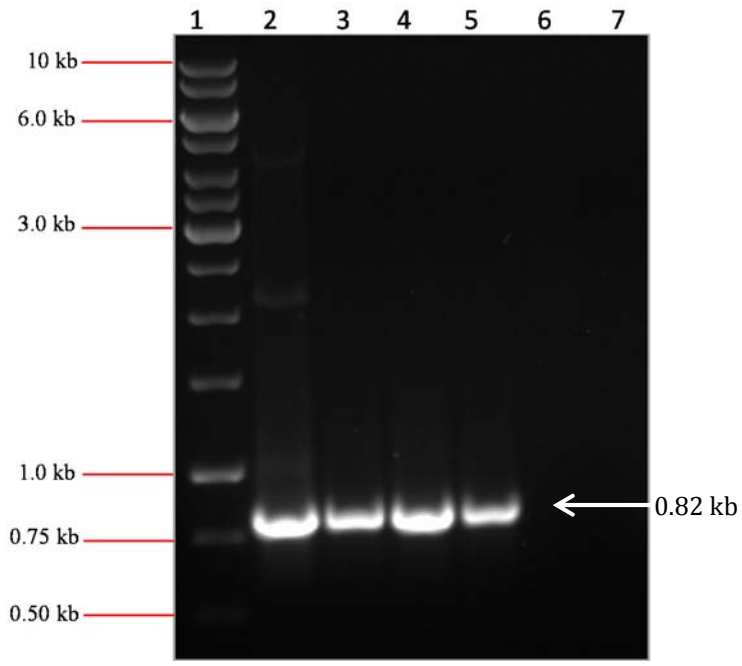


Figure 3. 9. Detection of plasmid pKluxCat in trans-conjugant V. midae colonies by PCR analyses. Lanes 1) 1kb DNA ladder, 2) plasmid pCAT19 DNA (positive), 3) pKluxCat from *V. midae* SY9 K811 Col 1, 4) pKluxCat from *V. midae* SY9 K811 Col 12, 5) pKluxCat from *V. midae* SY9 K811 Col 16, 6) Plasmid pAKlux2 DNA (negative) and 7) NTC. The white arrow on the right indicates PCR amplification products, that corresponds to the size of the *cat* gene (~ 0.82 kb).

3.3.2 Marker stability

After repeatedly sub-culturing *V. midae* SY9 K811 colonies every 24 hours onto TSA media supplemented with antibiotics, all 100 colonies grew in the presence of chloramphenicol and streptomycin following the first three passaging cycles. After the fourth passaging cycle, only 71 (71%) of the 100 colonies were able to grow on the selective media (Fig. 3.10). There was also a steady decline in bioluminescence signal from the colonies after repeated subculture on selective media, with the average bioluminescence intensity dropping from 2.31 x 10^8 photons per second (phot.sec^{-1}) after the first passaging cycle to 5.68 x 10^7 phot.sec^{-1} after the fourth passaging cycle (Fig. 3.11 A). Furthermore, by the fourth passage, 12% of the colonies (9 of the 71 remaining colonies) growing on the selective media no longer generated a detectable bioluminescent signal in the Xenogen (Fig. 3.11 B). These non-bioluminescent colonies were however capable of growing on the antibiotic supplemented media, indicating that they still retained the plasmid.

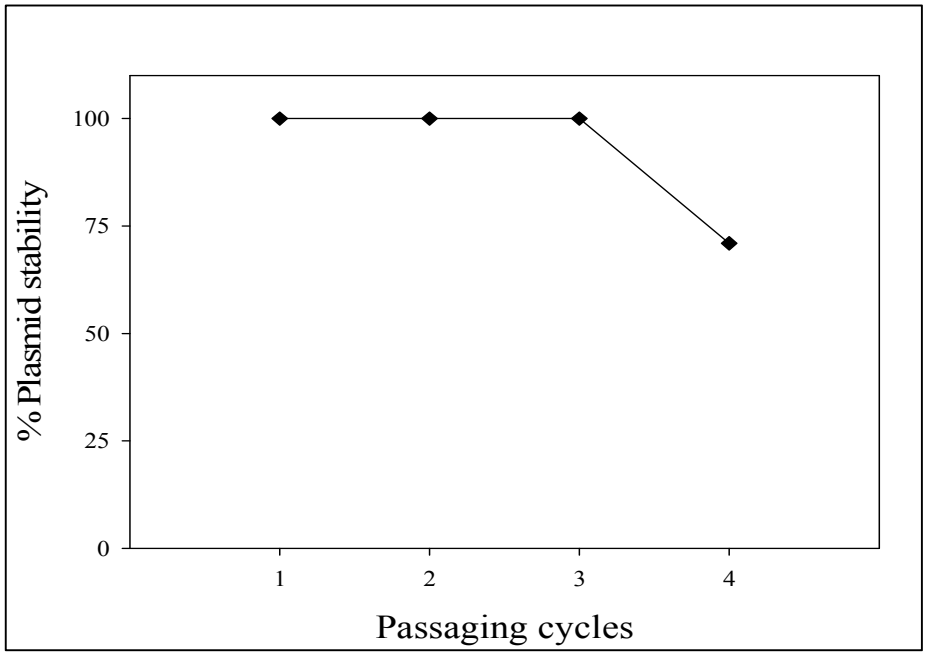


Figure 3. 10. *In vivo* stability of bioluminescent plasmid pKluxCat. The stability of the plasmid was determined by the percentage of colonies that grew on the selective plates compared to the number of colonies that grew on the non-selective plates. One passaging cycle represents 24hours.

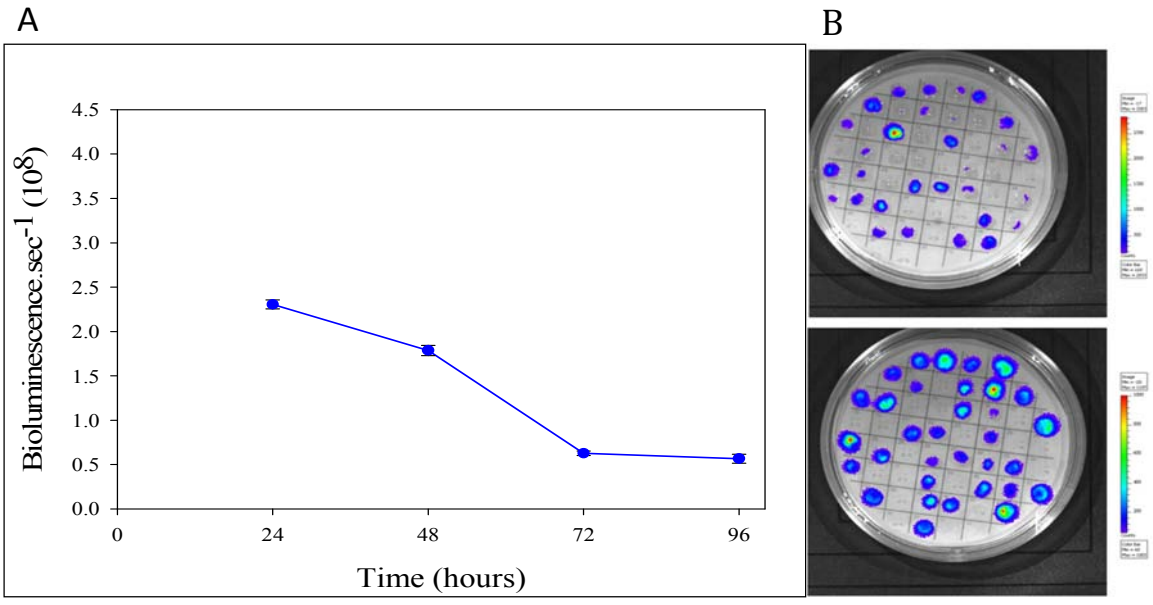


Figure 3. 11. The i*n vivo* bioluminescence activity of *V. midae* SY9 K811 colonies replica plated on TSA selective media every 24 hours. A) The bioluminescence emitted by the colonies was measured by placing the agar plates into the Lumina II imaging system, and the average bioluminescence calculated at each time point. B) Bioluminescent *V. midae* SY9 K811 colonies plated on selective media after the fourth transfer. One hundred colonies where split across 2 agar plates to account for the swarming property of this *Vibrio* strain.

In contrast, when *V. midae* SY9 K811 colonies were transferred to non-selective media, there was a 10-fold decrease in bioluminescence emission after the first passage when compared to cells plated on selective media (Fig. 3.12). Furthermore, bioluminescent signal was no longer detectable after 3 passages (Fig. 3.12 A and Fig. 3.13).

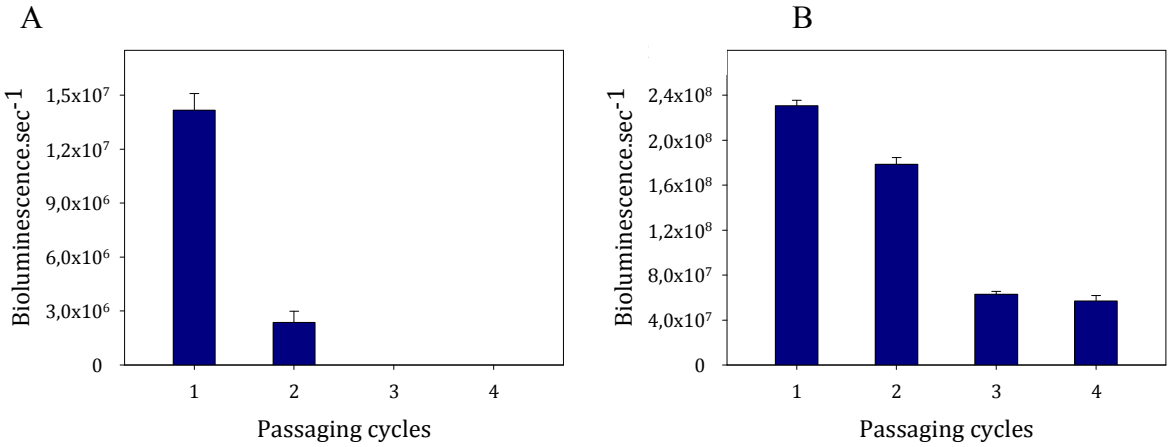


Figure 3. 12. Bioluminescence emitted by *V. midae* SY9 K811 transconjugant colonies. A) Bioluminescence emission from colonies plated on antibiotic free media. B) Bioluminescence emission from colonies plated on antibiotic supplemented media. The data represents the mean bioluminescence ± standard error

3.4 Growth curve analysis of *V. midae* SY9 K811

The growth profile and maximum specific growth rate (μ_{max}) of trans-conjugant *V. midae* SY9 K811 (1.021 h^{-1}) was similar to that of *V. midae* SY9 (1.024 h^{-1}) when cultured in media without antibiotic supplementation (Table 3.2, Fig. 3.13). Both strains lacked a distinct lag phase and entered the logarithmic (log) growth phase as early as 1 hour post-inoculation. The only notable difference between the two strains was that the wild-type strain reached a slightly higher maximum biomass yield compared to the trans-conjugant.

In contrast, *V. midae* SY9 K811 displayed a distinct lag phase and only entered log phase three hours post-inoculation when cultured in TSB media supplemented with antibiotics (Fig. 3.13). However, once the culture reached log phase, the growth profile of the trans-conjugant was comparable to that observed in the absence of antibiotic supplementation, reaching an equivalent biomass. When comparing the μ_{max} of *V. midae*

SY9 K811 cultured with antibiotics to that of the trans-conjugant and wild type strain cultured in antibiotic free media, a lower μ_{max} value (0.74 h^{-1}) was noted (Table 3.2).

Table 3. 2. Maximum specific growth rate of *V. midae* SY9 and *V. midae* SY9 K811 cultured in tryptic soya broth (TSB) with and without chloramphenicol and streptomycin.

Strain	**Media**	**Maximum specific growth rate (μ_{max})**
V. midae SY9 wt.	TSB only	1.024 h^{-1}
V. midae SY9 K811	TSB only	1.021 h^{-1}
V. midae SY9 K811	TSB + Cm + Sm	0.74 h^{-1}

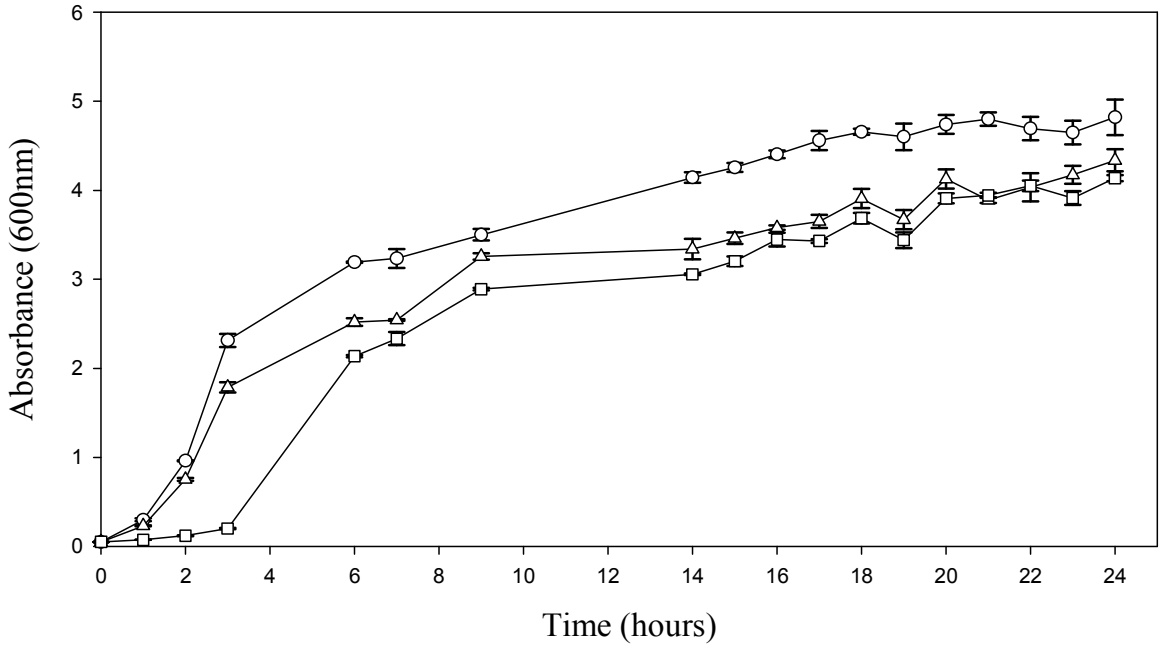


Figure 3. 13. Growth profile of *V. midae* SY9 and trans-conjugant *V. midae* SY9 K811 cultured in TSB media with and without streptomycin and chloramphenicol antibiotics. V. *midae* SY9 wt cultured in TSB media without antibiotics (○). *V. midae* SY9 K811 cultured in TSB without antibiotics (△). *V. midae* SY9 K811 cultured in TSB supplemented with streptomycin and chloramphenicol antibiotics (□). The data represents the means ± standard error (n=3)

When comparing the bioluminescent profiles of *V. midae* SY9 K811 cells cultured in media with and without antibiotics, it was noted that both cultures had analogous profiles, demonstrating bioluminescence emission in the lag phase of growth (Fig. 3.14).

However, the bioluminescence profile for both cultures was very different after the first 3 hours of growth.

V. midae SY9 K811 inoculated into antibiotic-free media showed a steady increase in bioluminescence immediately after inoculation, with bioluminescence peaking at 3 hours (3.38×10^6 phot.sec^{-1}) during the logarithmic phase of growth (Fig 3.14 A). Thereafter, bioluminescence steadily decreased to a minimum of approximately 0.2×10^6 phot.sec^{-1}, where it remained for the duration of the experiment (up to 24 hours, Fig. 3.14 A) In contrast, *V. midae* SY9 K811 cultured in TSB media supplemented with antibiotics exhibited a steady increase in bioluminescence which peaked at six hours (1.79×10^8 phot.sec^{-1}) during logarithmic phase of growth. Thereafter, bioluminescence decreased steadily to around 8.97×10^7 phot.sec^{-1} immediately after the culture entered stationary phase of growth (13 hrs, Fig. 3.14 B). However, the levels of bioluminescence were significantly higher ($P<0.001$) compared with cells grown in non-antibiotic supplemented media and remained as high as 8.35×10^7 phot.sec^{-1} at 24 hours of growth.

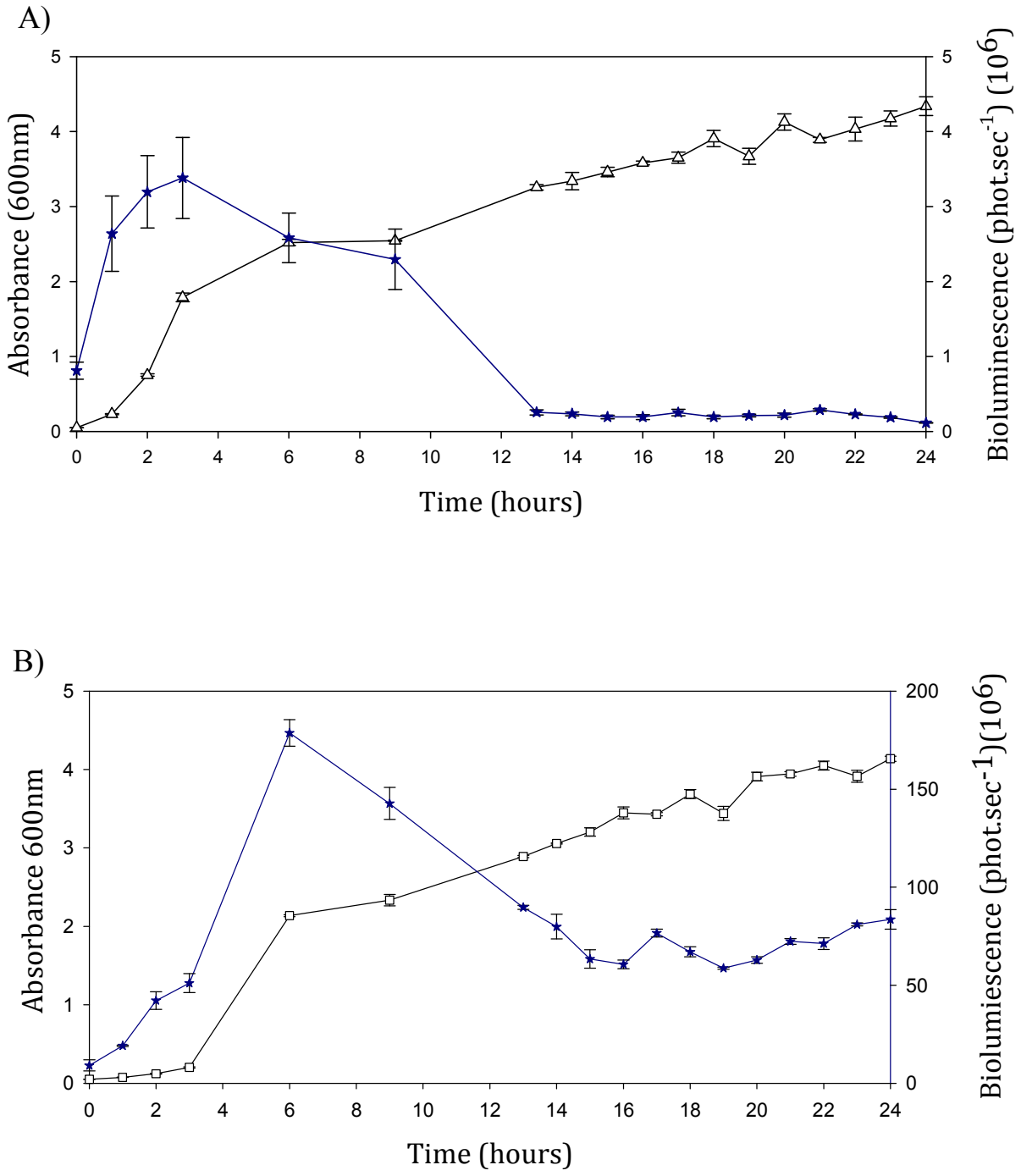


Figure 3. 14. Growth and bioluminescence profile of *V. midae* SY9 K811 shake flask cultured for 24 hours. A) Growth profile (△) and bioluminescence emission (★) for *V. midae* SY9 K811 cultured in TSB media without antibiotics. B) Growth profile (□) and bioluminescence (★) emission of *V. midae* SY9 K811 cultured in TSB media with antibiotics. The data represents the means ± standard error (n= 3).

3.4.1 Investigation of the decreased bioluminescence in *V. midae* SY9 K811 cultured in the absence of antibiotics

The large decrease in bioluminescence detected for *V. midae* SY9 K811 cultured in the absence of antibiotics was investigate to determine whether the decreasing signal was as a result of plasmid loss.

The decrease in bioluminescence observed in the absence of antibiotics was correlated with a loss of the plasmid pKluxCat. *V. midae* SY9 K811 grown in the absence of antibiotics and subsequently plated onto selective and non-selective media showed a 100 to 1000 fold decrease in bacterial cell numbers on the selective media when compared with non-selective media (Fig. 3.16). By 16 hours, the number of colony forming units on selective plates was 2.03 x 10^7 cfu.ml^{-1}, compared with 10^9 cfu.ml^{-1} on non-selective plates (Fig. 3.16 A). All of the colonies present on the non-selective media were no longer bioluminescent. Furthermore, PCR using the Tn9 *cat* gene primer set confirmed the absence of plasmid pKluxCat as indicated by the lack of the 824 bp *cat* gene PCR product (Fig. 3. 17).

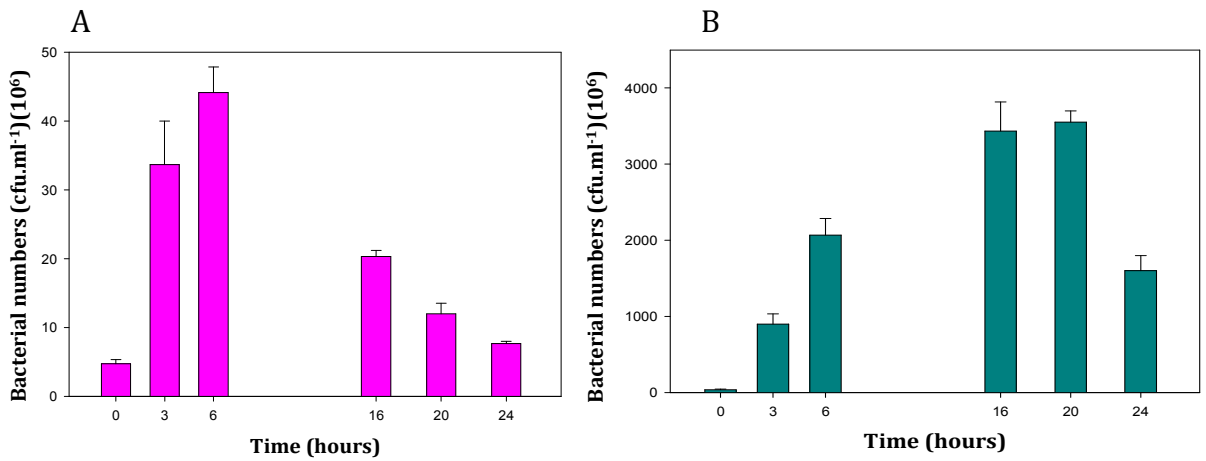


Figure 3. 15. Bacterial colony forming units for samples of *V. midae* SY9 K811 cultured in TSA media without antibiotics. Bars represent A) the number of *V. midae* SY9 K811 colonies present on selective (■) (TSA + chloramphenicol + Streptomycin) and B) the number of *V. midae* SY9 K811 colonies presnt on non-selective (■) (TSA only) media. Error bars represent standard error (n=3).

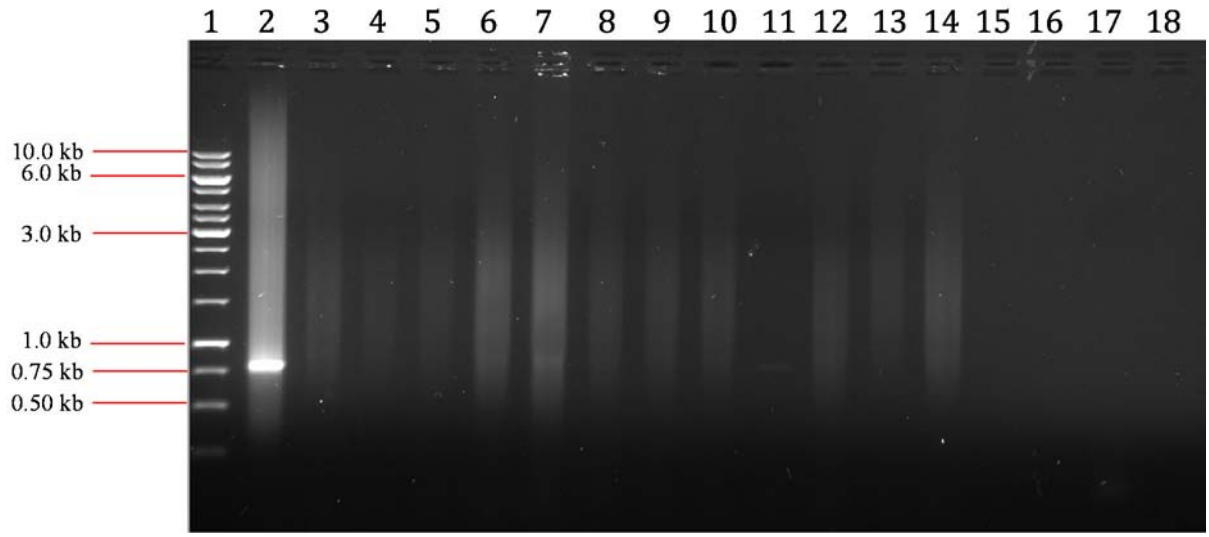


Figure 3. 16. Colony PCR for *V. midae* SY9 K811 cells cultured in the absence of antibiotics and plated on non-selective TSA media. Lanes :1) 1kb DNA marker, 2) pCAT19 plasmid DNA (positive control), 3-5) *V. midae* SY9 K811 sampled after inoculation (T= 0), 6-8) *V. midae* SY9 K811 sampled at 3 hours of growth ,9-11) *V. midae* SY9 K811 sampled at 6 hours of growth 12-14) *V. midae* SY9 K811 sampled at 14 hours of growth 15- 17) *V. midae* SY9 K811 sampled at 24 hours of growth,18) No template control (NTC).

3.5 *In vivo* detection of bioluminescent *V. midae* SY9 K811 bacterial cells

The relationship between bacterial cell number and bioluminescence emission was investigated to determine the lower limit of bioluminescence detection for this bacterial strain using the Xenogen. It was also investigated in order to correlate bioluminescence levels with bacterial cell number.

There was a strong linear correlation between bioluminescence (phot.sec^{-1}) and viable cell counts (cfu.ml^{-1}) (r^2 = 99) (Fig. 3.18), indicating that photon emission, detected by the Xenogen and quantitated using Living image software, accurately reflects the number of viable *V. midae* SY9 K811 bacterial cells. The minimum number of bioluminescent *V. midae* SY9 K811 cells required for accurate detection of bioluminescence *in vivo* was found to be 5.264 x 10^4 cfu.ml^{-1}.

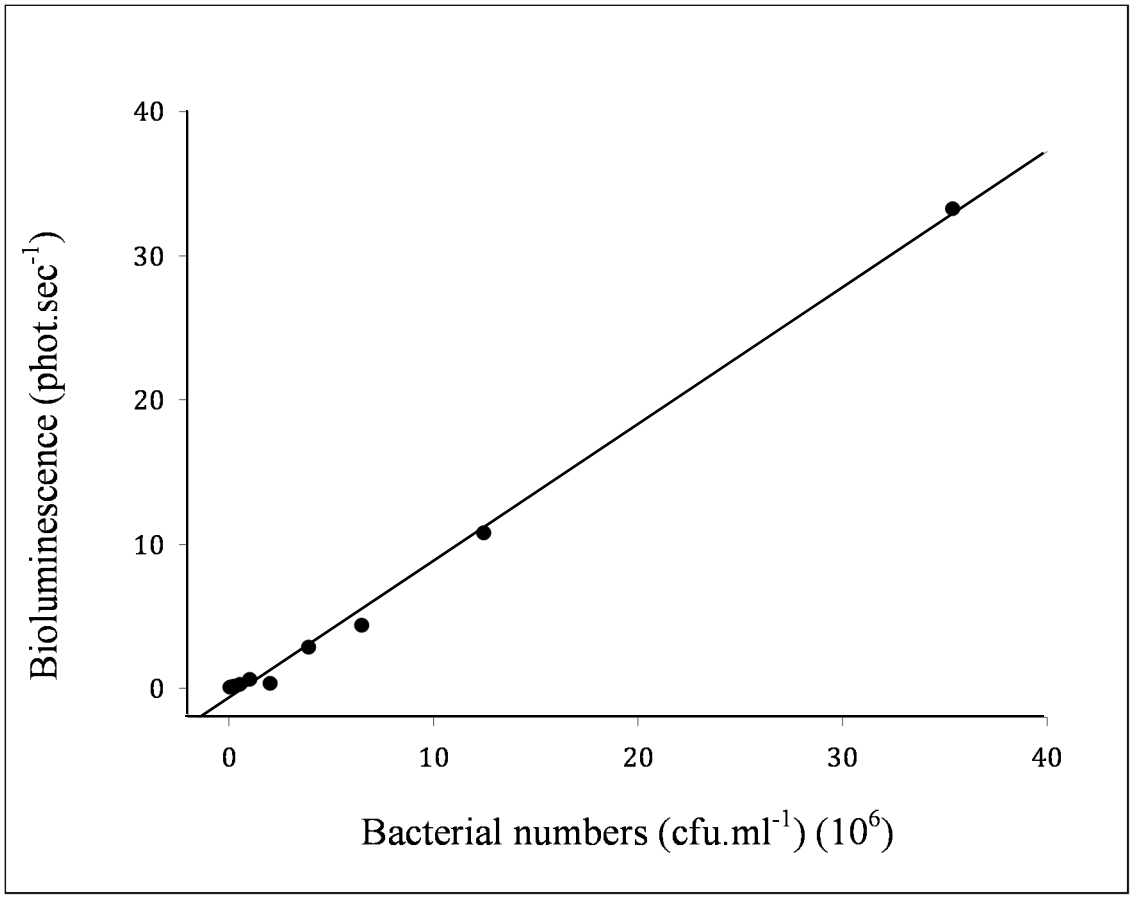


Figure 3. 17. Correlation between mean bioluminescence (phot.sec^{-1}) and mean *V. midae* SY9 K811 cell number (cfu.ml^{-1}). (r^2 = 0.99).

3.6 *In vivo* detection of bioluminescent *V. midae* SY9 K811 administered to juvenile *Haliotis midae* abalone.

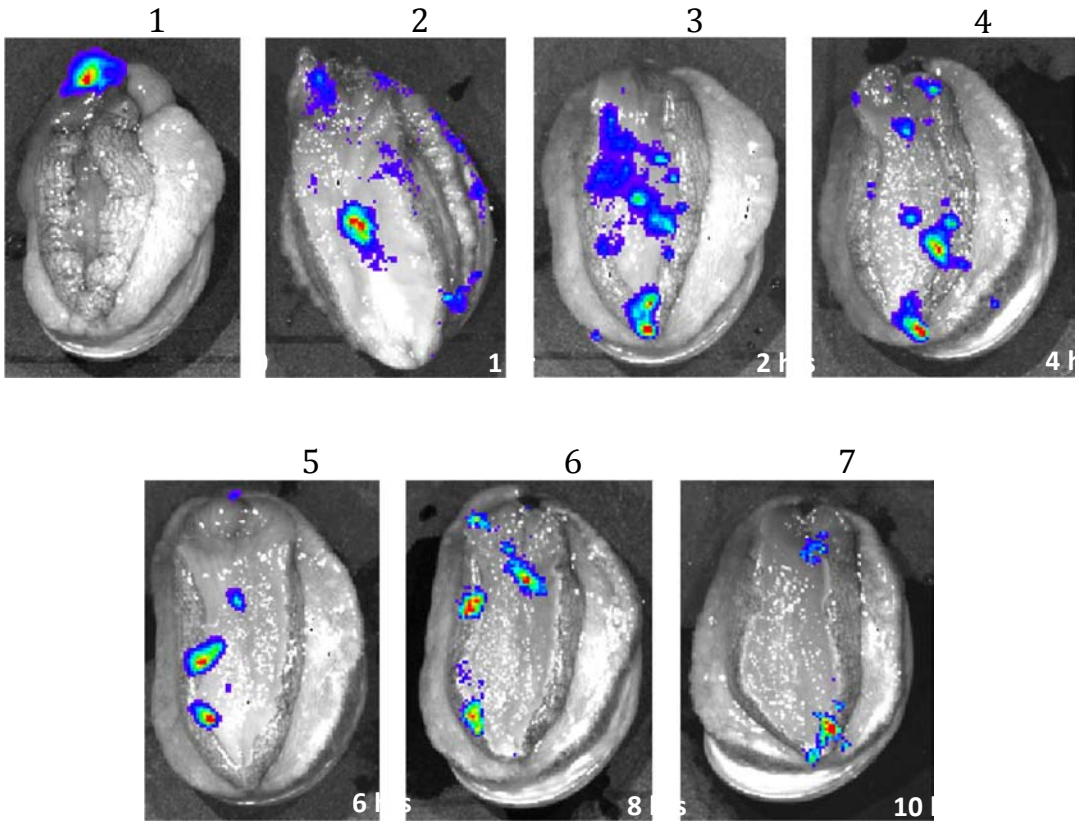
To detect *V. midae* SY9 non-invasively within the digestive tract of South African abalone *Haliotis midae,* the bioluminescent bacterial strain was administered to juvenile abalone by oral gavage.

Following oral administration of a defined dose (7.8 X 10^7 cfu.ml^{-1}) of *V. midae* SY9 K811, bioluminescent bacterial cells were detected within the digestive tract of the juvenile abalone for up to 10 hours (Fig. 3.19), while no signal was detected in control animals. However, the amount of bioluminescence detected within abalone tissue over time was highly variable (Fig 3.19). In general, a strong bioluminescent signal was

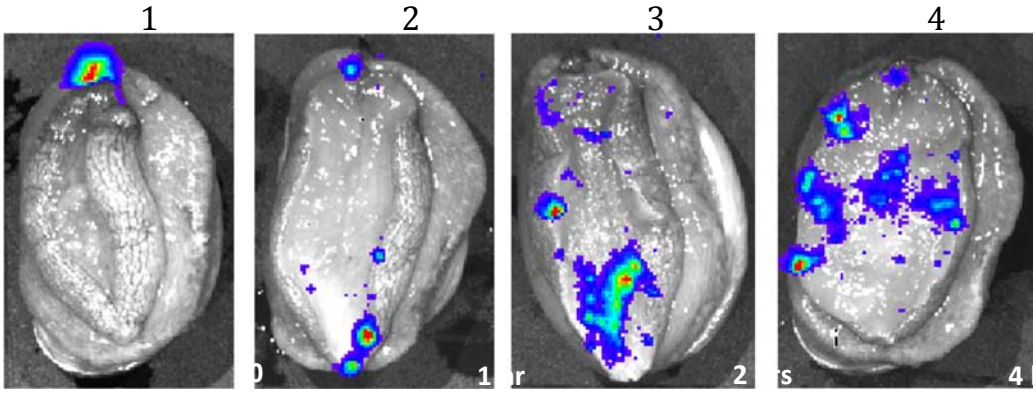
detected close to the mouth of the abalone immediately after oral administration of the bacterial suspension (Fig. 3.19 A1, B1 and C1). Subsequent images suggest that the bacteria move away from the mouth towards distal regions of the abalone digestive tract (Fig. 3.19 A 2-7, B 2-7 and C 2-5). However, the areas in which the bacterial cells were identified cannot conclusively be stated, as some of the bioluminescent areas did not correspond to any part of the abalone digestive tract.

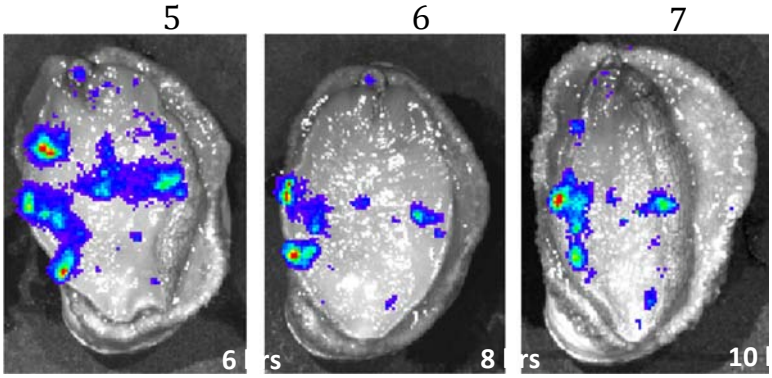
The average total level of bioluminescence from an entire animal recorded immediately after inoculation of the labelled bacterial strain was 3.73 x 10^7 phot.sec^{-1} (Fig. 3. 20). Thereafter, the bioluminescent signal decreased significantly to 2.8 x 10^6 phot.sec^{-1} by 2 hours post-inoculation. Subsequent imaging at time points 4, 6, 8 and 10 hours post-inoculation demonstrated a steady, but significant decrease ($p < 0.05$) in bioluminescence when compared to time 0, until signal was no longer detectable at 12 hours post-inoculation.

A)

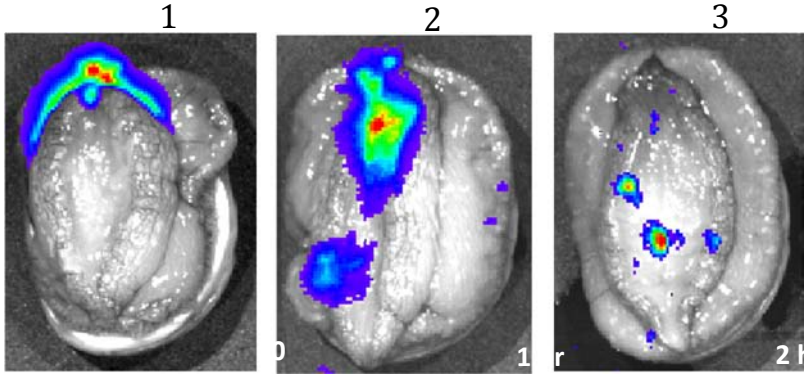


B)





C)



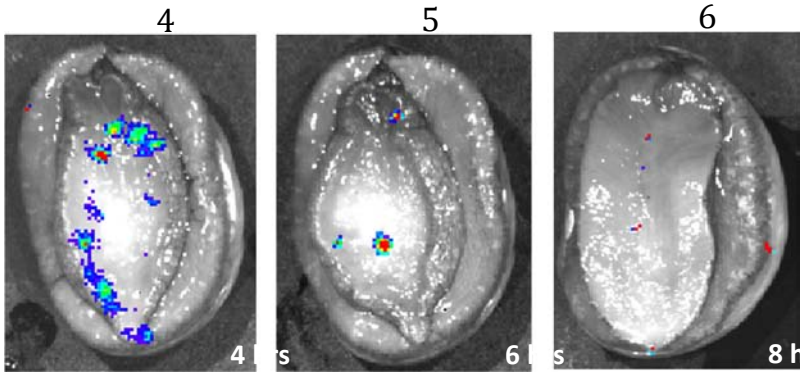


Figure 3. 18. Xenogen images of abalone inoculated with bioluminescent *V. midae* SY9 K811 cells. A, B and C represent three of the 12 animals that were imaged in the Xenogen at various time points post-administration. The pseudocolour images indicate the presence of bioluminescent bacterial cells.

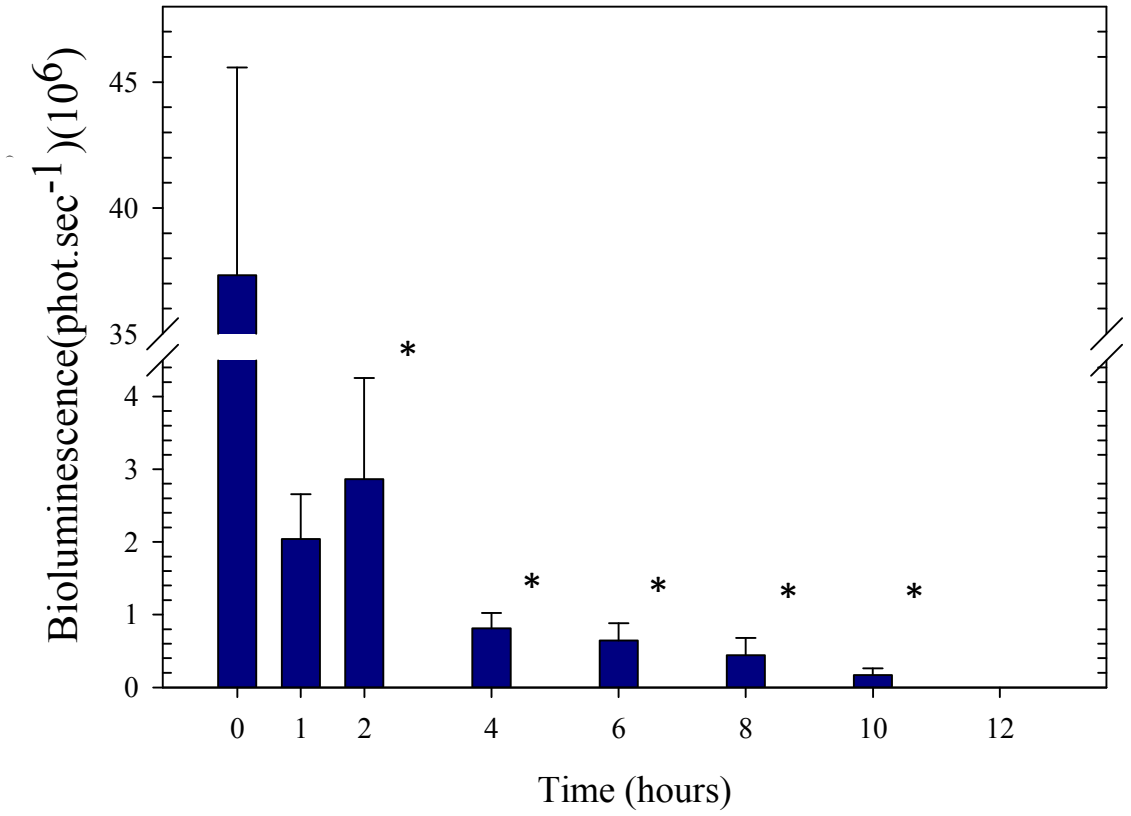


Figure 3. 19 Bioluminescence measured in *Haliotis midae* abalone exposed to *V. midae* SY9 K811 by oral gavage. Animals were viewed in the Xenogen at 0, 1, 2, 4, 6, 8, 10 and 12 hours post inoculation of strain SY9 K811, and were imaged for 1 min. The data represents the mean bioluminescence ± standard error (n = 12). The asterix (*) represents bioluminescent values that are significantly different (Tukey Test; $p<0.05$) to the measurement captured at time 0.

Chapter 4

Discussion

4.1 Introduction

In the fourth century BC, the Greek philosopher Aristotle recognised that bioluminescence was a process of light emission without heat (Meighen, 1988). Bioluminescence is a biological process catalysed by luciferase enzymes that generate light through oxidation of an enzyme-specific substrate in the presence of oxygen (Contag & Bachmann, 2002). The *luc* gene from the North American firefly *Photinus pyralis* (Contag & Bachmann, 2002; Hakkila *et al.*, 2002,) and bacterial *lux* genes (*lux*CDABE) belonging to species such as *Vibrio harveyi*, *Vibrio fischeri* and *Photorhabdus luminescens* (Hakkila *et al.*, 2002), are some of the more frequently utilised luciferase genes. The range of bioluminescent reporter systems available, coupled with the emerging field of charge-coupled device (CCD) camera technology, has enabled the detection of light through animal tissue and has given rise to the technique known as bioluminescent imaging (BLI).

BLI has been recognised as a powerful method for real time investigation of a whole host of biological activities which include processes such as disease progression, gut-pathogen interactions, bacterial colonisation and gene regulation (Contag & Bachmann, 2002; Foucault *et al.*, 2010). BLI was therefore chosen as the imaging method to non-invasively investigate the movement of bioluminescent *V. midae* SY9 within the digestive tract of juvenile abalone, allowing for easy detection of the target bacterium in a mixed population of bacteria. In order to achieve this, plasmid pKluxCat, carrying the *P. luminescens lux* operon, was used to label the probiotic bacterium *V. midae* SY9. The bacterium was successfully labelled with the luciferase expression vector using a conjugal mating technique, and the plasmid-harbouring cells were able to constitutively emit bioluminescence. The plasmid proved to be stable within *V. midae* SY9 and growth curve analysis demonstrated that carriage of plasmid pKluxCat had no effect on the growth of these cells *in vitro*. Administration of the labelled *V. midae* SY9 cells to *Haliotis*

midae abalone resulted in non-invasive visualisation of bioluminescence within the animals.

4.2 Construction of plasmid pKluxCat

Plasmid pAKlux2, which contains an ampicillin resistance gene (*bla*) and constitutively expresses the bacterial *lux*CDABE genes, was constructed by Karsi and Lawrence (2007) as a broad host range bioluminescence expression vector for Gram-negative bacteria. The construct was selected to label the Gram-negative probiotic bacterium *V. midae* SY9 as light emission does not require the addition of a substrate. Another advantage of this bioluminescent system is that light emission is dependent on substrate provided by cellular metabolism and therefore provides a direct indication of cell viability. Initially, an attempt was made to label *V. midae* SY9 with pAKlux2. However, differentiating labelled *V. midae* cells from the *E. coli* donor and unlabelled *V. midae* cells proved difficult on ampicillin selective media. This phenomenon however is not uncommon as Sawabe *et al.* (2006) reported that it is often difficult to distinguish labelled *Vibrio* recipient cells from their *E. coli* donors. The method used to specifically label *Vibrio* bacteria was an adaptation of the conjugal mating technique described by Sawabe *et al.* (2006). However, selection of positive transformants was not possible due to the discovery that *V. midae* was highly resistant to ampicillin (unpublished data). The resistance of *V. midae* SY9 to ampicillin was supported by the work of Lin *et al.* (1999), who reported that 60.8% of marine *Vibrio* strains isolated from silver sea bream (*Sparus sarba*) were highly resistant to ampicillin. This necessitated the addition of an alternate selection marker to try and separate the labelled recipient cells from their *E. coli* donors. In various studies conducted using *Vibrio* strains, it was found that a large variety of species were highly sensitive to chloramphenicol (Backhaus & Grimme 1998; Lin *et al*., 1999). Based on these findings, wild type *V. midae* SY9 was exposed to varying concentrations of the antibiotic and found to be highly sensitive to chloramphenicol. Consequently, a Tn9 chloramphenicol acetyltransferase (*cat*) gene, encoding resistance to the chloramphenicol antibiotic, was cloned into plasmid pAKlux2.

The Tn9 *cat* gene from plasmid pCAT19 was excised and cloned into pAKlux2 resulting in a new plasmid construct named pKluxCat. The new luciferase expression vector now

contained the constitutively expressed *lux* operon as a well as both ampicillin and chloramphenicol antibiotic resistance genes. Transfer of pKluxCat into *V. midae* SY9 SR11 (streptomycin resistant strain) through the process of conjugal mating yielded *Vibrio* transconjugants that grew on chloramphenicol supplemented media, indicating that they were no longer sensitive to the antibiotic. The transconjugant cells also emitted bioluminescence without the addition of an inducer (IPTG), indicating that the lux operon was constitutively expressed and functional, which is in accordance to the study conducted by Karsi and Lawrence (2007).The presence of plasmid pKluxCat within these bacterial cells was validated by PCR analysis. Once it had been confirmed that pKluxCat had been successfully conjugated into *V. midae* SY9, it was important to investigate the stability of the plasmid within the bacterial cells.

4.3 Plasmid stability

Bioluminescence imaging has been used as a tool to monitor and measure processes such as *in vivo* infections, bacterial colonisation and drug efficacy. Some of these processes have only involved short term monitoring as long term studies call for stable maintenance of the plasmids (Foucault *et al*., 2010) which usually requires antibiotic selection. However, constant selection pressure is not feasible as this would require the abalone to be exposed to antibiotics which could impact the host animal. For instance, in the case of an infection study, antibiotics may interfere with the infection process being monitored and may affect intestinal colonisation by the bacterium (Foucault *et al*., 2010). The presence of antibiotics may also be lethal to the antibiotic sensitive native microflora of the host. For this study, retention of plasmid pKluxCat by *V. midae* SY9 K811, as well as bioluminescence emission, was monitored to determine the stability of the plasmid in the absence of antibiotic selection.

In a study conducted by Foucault *et al.* (2010), it was noted that the bioluminescent plasmid pSB2025 (containing the *P. luminescens lux* operon) only remained stable during the first two days of culture, after which rapid plasmid loss was observed. Plasmid loss correlated with a loss in bioluminescence, which occurred with each successive transfer. In this study, plasmid pKluxCat remained stable in *V. midae* SY9 K811 for the first three passages, while bioluminescence gradually declined following

each successive transfer of the colonies onto fresh selective media. After the fourth passage, 71 trans-conjugant colonies remained on the antibiotic supplemented media, of which 12% were no longer bioluminescent. The ability of the bioluminescent negative bacterial colonies to grow on the antibiotic supplemented agar plates indicated that these cells still harboured plasmid pKluxCat. This demonstrated that the decrease in bioluminescence did not coincide with plasmid loss, which is contrary to what was found by Foucault *et al.* (2010).

Initially, it was thought that insertion of the cat gene into the pAKlux2 vector could have potentially affected expression of the *lux* genes. This would explain why the colonies grew on the selection media but were not bioluminescent. However, sequencing of pKluxCat proved that the chloramphenicol gene was cloned into the three prime end of the *lux* operon and therefore was not disrupting the *lac*Z promoter or any of the *lux* genes. This result was further supported by the restriction enzyme digest profiles of plasmids pCAT19 and pAKlux2 (during construction of pKluxCat), which demonstrated that the Tn9 *cat* gene was correctly excised from pCAT19 and inserted in the correct orientation into pAKlux2. The expression of the *lux* operon, and subsequent bioluminescence, places a large metabolic burden on the bacterial cells as it requires oxygen and metabolic components (such as nucleotides, amino acids etc.) from the bacterium (Georgiou, 1988). This makes it quite possible that only the *cat* gene was expressed with each transfer of the colonies onto fresh selective media due to selective pressure exerted by the presence of chloramphenicol in the agar. To our knowledge, the loss in bioluminescence and retention of antibiotic resistance has not previously been reported, warranting further investigation as we are uncertain as to why this occurred. This could be further investigated by isolating the plasmid and performing PCR and RE digest analyses to determine whether the *lux* genes are still present on the plasmid. To determine whether the lux gene have been silenced, resulting in the absence of bioluminescence, quantitative real time PCR analyses could be conducted

4.4 Growth profiles of bioluminescent *V. midae* SY9 K811

The survival of a plasmid-harbouring bacterium can be affected by the presence of the extra-chromosomal element (Gowland & Slater, 1984), as synthesis of plasmid-encoded

products may place a metabolic burden on the host. The fitness of plasmid-containing bacterial cells can be influenced by various factors, one of which includes differing growth conditions, such as media composition or the absence of plasmid selection. This has been shown to potentially cause changes in transformed cells and affect the growth of these strains (Smith & Bidochka, 1998). The growth profile and maximum specific growth rate (μ_{max}) of the *V. midae* SY9 K811 transconjugant strain was compared to that of the wild-type to determine whether plasmid pKluxCat had any effect on the growth of these bacterial cells in the presence and absence of antibiotic selection.

According to Lenski (1998) a resistant bacterial strain may have a lower growth rate compared to the wild-type or sensitive strain in the absence of antibiotic selection. In this study, *V. midae* SY9 K811 and *V. midae* SY9 wild-type displayed analogous growth kinetics with almost identical maximum specific growth rates in the absence of antibiotic selection. This indicated that plasmid pKluxCat did not place a metabolic burden on the transconjugant cells in the absence of antibiotic selection (Beard *et al*., 2002). This observation was significant since the labelled cells would be administered to abalone in an antibiotic-free environment.

When comparing the growth rate of *V. midae* SY9 K811 cultured in antibiotic-supplemented media to that of the wild-type strain, a noticeable difference in the growth profiles of the two strains was observed. The transconjugant strain had a distinct lag phase and its maximum specific growth rate was markedly lower than both the wild-type and the transconjugant strain cultured in the absence of antibiotics. This is in accordance with a study conducted by Smith and Bidochka (1998) who demonstrated that in the presence of selective pressure, in comparison to antibiotic-free media, the lag phase of bacterial strains carrying a plasmid is extended. The longer lag phase can be explained by the fact that cells that harbour plasmids are obligated to synthesise more DNA, mRNA and proteins which causes an increase in energy consumption by the cell (Georgiou, 1988). This usually means that the culture takes a longer time to reach the logarithmic phase of growth as more cellular components need to be synthesized (Kumar *et al*., 1991). Although the maximum specific growth rate of the transconjugant cultured with antibiotics was lower compared to culturing the strain without antibiotics, both strains eventually reached the same cell mass and displayed

analogous growth profiles. This once again indicated that plasmid pKluxCat did not affect the growth of *V. midae* SY9 cells and that it could successfully be cultured with or without antibiotic selection.

4.5 Bioluminescent profiles of *V. midae* SY9 K811 cultured with and without antibiotic selection

Although analysis of the growth profile of *V. midae* SY9 K811 cultured in the absence of antibiotic selection demonstrated that the presence of plasmid pKluxCat had no effect on the growth of these cells, it was necessary to investigate the effect that antibiotic absence had on the bioluminescent profile of this bacterium. Plasmids often become redundant in the absence of selection and this causes them to become unstable, fragment or be completely eliminated (Goodwin & Slater 1979; Jones *et al*., 1980). It was also essential to monitor bioluminescence in an antibiotic-free culture, as the strain would be fed to live abalone in the absence of antibiotics.

The ability of bioluminescent bacterial cells to emit light is dependent on the stage of growth or age of the culture, as bioluminescence emission is highly dependent on the metabolic state of the cells (Doyle *et al*., 2004; Karsi and Lawrence, 2007; Tamagnini *et al*., 2008). *V. midae* SY9 K811 cultured in antibiotic-supplemented media demonstrated a steady increase in bioluminescence which corresponded to the logarithmic growth phase of the culture. It is at this stage of growth that the cells are actively metabolising, therefore providing the necessary substrate required for bioluminescence emission. A gradual decline in light emission was observed as the culture entered stationary phase. This was expected as it has been demonstrated that for many bacterial species harbouring the *lux* operon, bioluminescence declined as the cells entered stationary phase during *in vitro* growth (Francis *et al*., 2001; Wiles *et al*., 2004). A decrease in bioluminescence is usually as a result of decreased metabolism during this phase of growth (Beard *et al*., 2002), which limits substrate availability. These results demonstrated that bioluminescence was steadily maintained in the presence of antibiotics and that emission was at its highest when the culture was in the log phase of growth.

The bioluminescence of the transconjugant strain cultured in the absence of antibiotics increased exponentially over the first three hours of growth after which it steadily decreased, even though the culture was still in log phase. This was unexpected as the cells were still actively growing; data obtained for the strain grown in the presence of antibiotics demonstrated that a decrease in bioluminescence only occurred as metabolism decreased during stationary phase. At the end of the growth period, bioluminescence emission was significantly less than that of the culture grown in the presence of antibiotics. Such a significant decrease in bioluminescence may be attributed to plasmid loss, which could have occurred due to a lack of selective pressure as a consequence of the absence of chloramphenicol. Indeed, PCR analysis of colonies cultured on antibiotic-free media confirmed that plasmid pKluxCat was no longer present in the transconjugants, therefore accounting for the decreasing amounts of bioluminescence detected.

Plasmid loss occurs for many reasons, one of which is plasmid copy number. It has been demonstrated that the number of cells harbouring low copy number plasmids, such as pKluxCat, decrease in antibiotic-free media (absence of selective pressure) due to plasmid segregation at cell division (Smith & Bidochka, 1998). Since partitioning of plasmid molecules into daughter cells after cell division is a random event, where a daughter cell could inherit 0 to *n* plasmid molecules, this phenomenon is more prevalent with low copy number plasmids as cell division does not always result in a daughter cell obtaining a copy of the plasmid (Boe *et al*., 1987).

Size is another property of plasmids that affects stable maintenance within a host cell. Maintenance of large plasmids usually places a metabolic burden on the bacterial host which is more prevalent during stationary phase when nutrient limitation problems occur. Smith and Bidochka (1998) demonstrated that under starved conditions, plasmid loss occurred in *E. coli* strains carrying plasmids of various sizes (2.916 to 11.916 kb). The loss correlated with the size of the plasmid such that the larger the plasmid, the greater the loss. Although it cannot conclusively be determined whether the copy number or size of pKluxCat affected its maintenance in *V. midae* SY9, it is likely that the plasmid was merely released due to the absence of selective pressure. Plasmid stability and bioluminescence emission was monitored in *V. midae* SY9 K811 grown on both solid media and in liquid media. Interestingly, colonies growing on solid media retained

the plasmid for a longer period in the absence of antibiotics. Thus, although the bacteria would be administered to the abalone in a liquid suspension, they would subsequently settle on solid surfaces (gut epithelium or particulate matter in the abalone digestive tract) and consequently, possibly maintain pKluxCat for an extended period.

4.6 *In vivo* monitoring of bioluminescent cells

Successful labelling of *V. midae* SY9 cells with plasmid pKluxCat resulted in a bioluminescent transconjugant strain that was chloramphenicol resistant. Characterisation of plasmid stability, growth and bioluminescent profiles of this strain indicated that the plasmid was stably maintained although plasmid loss did occur to some degree in the absence of antibiotic selection. These results indicated that short term monitoring of this strain in abalone using BLI was possible. Although BLI has been efficiently employed to investigate the relationship between bacterial cells and their host (Contag *et al*, 1995; Wiles *et al*., 2006; Bina *et al.*, 2010; Gonzalez *et al*., 2012; Plaut *et al*., 2013), most of these studies have been conducted using a murine model. To our knowledge, this is the first reported study using BLI coupled with CCD technology to investigate bacteria-host association in a live invertebrate system, and more specifically in a *Haliotis midae* abalone model.

V. midae SY9 is a probiotic bacterium that was isolated from the gut of *H. midae* abalone. Studies conducted using this bacterial strain demonstrated that it had the ability to colonise the gastrointestinal tract of this species of abalone (Macey & Coyne, 2005; Huddy & Coyne 2014). A suspension of bioluminescent *V. midae* SY9 K811 was administered to juvenile abalone by oral gavage at a cell number of 7.8×10^7 cfu.ml^{-1}. The administered dose was in accordance with the concentration of strain SY9 administered to abalone in a previous colonisation study (Macey, 2005). Abalone were visualised in the Xenogen immediately after bacterial inoculation, where a strong bioluminescent signal was instantly detected. The signal was found to be localised in the area that corresponded to the abalone mouth region. Bioluminescence was not detected in animals that had not been inoculated with the bacterium. This demonstrated that abalone are bioluminescent negative and that the bioluminescent signal detected was due to the presence of bioluminescent *V. midae* SY9 K811. One hour post-gavage, a large

but statistically insignificant decrease in bioluminescence was detected in animals fed the bioluminescent strain. The drop in signal could have been due to numerous factors, including bacteria leaking out the mouth, being washed away once the animals were placed back in the tanks, or being cleared through mucous production (Harris *et al*., 1998a). Interestingly, the abalone foot was wiped with moist tissue paper to remove any debris prior to imaging the animals in the Xenogen. In so doing, a large amount of mucous (located around the sides of the foot) was removed. A strong bioluminescent signal was detected when the mucous collected on the tissue was analysed in the Xenogen, indicating the presence of *V. midae* SY9 K811 cells. It is therefore quite possible that the drop in bioluminescence 1 hour post-gavage was indeed due to clearance of bacteria through mucous production. The bioluminescence signal associated with the abalone increased significantly ($p<0.05$) two hours post-gavage which could potentially be due to bacterial cells entering the digestive tract and establishing themselves. In one of the abalone colonisation studies conducted with *V. midae SY9,* it was suggested that the bacterium possessed the ability to adhere to the mucous lining of the abalone gut (Huddy & Coyne, 2014). The increase in light emission one hour post gavage in the present study could therefore be due to the labelled strain establishing itself in the oesophageal region by adhering to the mucosal lining of the digestive tract.

Bioluminescence was found to steadily and significantly decrease at subsequent imaging intervals, and only remained detectable up to 10 hours post-gavage. This is contrary to what was found by Macey (2005) who demonstrated that the number of *V. midae* SY9 cells in the abalone digestive tract only decreased two days post-feeding with probiotic-supplemented feed. In the present study, abalone were fed macroalgae every three days over a two week period prior to oral administration of the bioluminescent bacteria. However, it was noted that the animals were not eating very well which could have been due to a number of factors, including poor water quality or stress due to water changes during the weekly tank cleaning process. Food was withheld for approximately 8 hours prior to oral administration of the *V. midae* SY9 K811 to the abalone as per standard practise, to ensure that the bacterial suspension would be efficiently ingested. Withholding food prior to experimentation, coupled with irregular intake of food by the abalone during the two week acclimatisation period, may have

resulted in a reduction in the retention time of *V. midae* SY9 K811 in the digestive tract as *V. midae* SY9 cells have been found to be associated with feed and other particulate matter in the abalone digestive tract (Macey, 2005; Huddy & Coyne, 2014). It is also important to note that Macey (2005) fed the abalone a probiotic-supplemented artificial feed whereas bacteria were orally administered in a liquid suspension in this study. In a study conducted to determine whether BLI could be used to monitor *Citrobacter rodentium* bacterial numbers within the digestive tract of live mice, researchers found that inoculation of the bacteria by oral gavage resulted in most of the bacteria passing straight through the gut (Wiles *et al.*, 2006). Although the digestive system of a mouse and an abalone are significantly different, the starvation period prior to bacterial administration could have resulted in little to no particulate matter within the gut, therefore allowing the bacterial suspension to easily pass straight through the digestive tract. In summary, the decreased bioluminescence could have been due to numerous factors, some of which include plasmid loss, bacterial death, clearance via mucous secretions or digestion of bacteria as a nutrient source (Harris *et al.,* 1998b) However, it is important to note that even though signal was not detected 12 hours post-gavage, it cannot conclusively be stated that *V. midae* SY9 K811 was no longer present.

Administration of bioluminescent *V. midae* SY9 K811 to juvenile abalone demonstrated that BLI could successfully and non–invasively be used to detect bacterial cells for up to 10 hours post-administration. In addition to monitoring the presence of a bioluminescent signal within the abalone, we were also interested in determining whether bacterial movement through the digestive tract could be monitored as a foundation for future colonisation studies. This is important as the ability of a bacterial strain to colonise the gastrointestinal tract of an organism is usually measured by collecting faeces samples or sacrificing the host animal and culturing gut content. These approaches however do not provide a direct indication of the site of colonisation within the digestive tract (Foucault *et al*., 2010). In order to track and investigate bacterial movement within abalone, images of animals fed bioluminescent *V. midae* SY9 K811 were captured using the Xenogen. Images taken at the various time points revealed possible movement of bacteria away from the mouth region, as indicated by detection of bioluminescence in other regions of the abalone body. The foot of each animal was gently wiped with moist tissue paper before imaging to ensure removal of any

particulate matter and excess mucous in which bacteria could become trapped. The results obtained therefore indicated that the detected bioluminescence originated from within abalone. Approximately two hours post-administration of *V. midae* SY9 K811, analysis of the pseudocolour Xenogen images revealed a bright bioluminescent signal present in the area of the abalone that possibly corresponds to the crop/stomach area. This is an exciting observation as it is comparable to a study conducted by Macey (2005) who demonstrated that *V. midae* SY9.8 was localised to the *H. midae* abalone digestive tract. Unfortunately the data obtained in this study is not unequivocal since it cannot conclusively be stated that the area in which the bioluminescence signal was detected is indeed the crop/stomach since bioluminescence was also detected in other areas of the abalone that do not correspond to any part of the digestive tract.

A study conducted with the abalone *Haliotis iris* found that digestion products are transferred into the circulatory system via the haemolymph (Ragg, 2003). Indeed, abalone tissue is directly bathed in haemolymph since abalone have an open vascular system (Ragg & Taylor, 2005). Consequently, it is possible that *V. midae* SY9 K811 cells (undigested) may have entered the circulatory system of *H. midae*, explaining why bioluminescence occurred in areas that are not associated with the digestive tract. This however warrants further investigation to obtain a better understanding of the *H. midae* circulatory system and its interaction with the digestive tract.

Conclusion

The use of bioluminescent imaging coupled with CCD technology has enabled investigation of various processes that researchers previously were unable to monitor in real time. This study was the first to demonstrate that bioluminescent *V. midae* SY9 could be identified within juvenile *Haliotis midae*. Bioluminescence was visualised in real time for up to 10 hours after administration. This research is currently in the preliminary stages and future work may require genetic engineering of *V. midae* SY9 to improve the stability of bacterial luciferase expression. In addition, it is necessary to repeat the study using a larger number of animals to strengthen the statistical analysis as well as the current findings. An important factor critical to the success of future studies is the improvement of the water system in which animals are maintained prior to experimentation. The laboratory in which the animals were kept did not have the facilities required for a flow-through sea water system. As a result, the tanks were therefore fitted with a continuous recirculation system which required fresh seawater to be obtained from an external source on a weekly basis. This required the water to be removed at each cleaning which could have affected the heath of the animals. The process used to administer the bacteria to the abalone may also need to be modified. The labelled bacterial cells were administered to the animals in a liquid suspension; it may be better to administer the bacterium by incorporation in artificial abalone feed in order to increase bacterial retention time. Despite the constraints present in this study, we were successfully able to utilise bioluminescence imaging, with the aid of CCD technology, to detect bioluminescent *V. midae* SY9 K811 cells within *H. midae* abalone in real time and non-invasively.

Chapter 5

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