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Evaluation of Two Culture-Independent Methods for Quantification of the Fraction of K-and r-Strategists within Water Microbial Communities

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Biotechnology
Submission date: May 2015
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Acknowledgements

This master project was performed at the Department of Biotechnology, Norwegian University of Science and Technology (NTNU).

I would like to thank my supervisor Olav Vadstein for the opportunity to be a part of this interesting project and for your outstanding guidance both theoretically and practically throughout the whole process. My best potential was brought out because of you. I would also like to show my gratitude to my co-supervisor Ingrid Bakke, for guidance in the lab and for always taking your time, even if you didn't have it. It saved my day many times and I am profoundly grateful. My appreciation also goes to all the supportive staff at the Department of Biotechnology, Norwegian University of Science and Technology (NTNU) for excellent help during my study.

Last, but not least, I would thank my family, friends and classmates for all your support. Special thanks to my friends and classmates for your encouragement and supportive words. I would not have made it without you.

Trondheim, May 2015

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Abstract

The r/K concept is a theoretical concept that can serve as a tool in evaluating the risk of invasion by pathogenic bacterial species in rearing of marine fish larvae. In rearing of marine fish larvae, a dominance of opportunistic, fast-growing r-strategists can result in poor survival of the fish. Dominance of slow-growing K-strategists have been shown to increase the survival and growth. The r-strategists generally exhibits many copies of the 16S rRNA gene in their genomes, whereas the K-strategists have one or a few. Moreover, the maximum specific growth rate also correlates with the maximum RNA content of a cell. Fast-growing r-strategists thus have a higher maximum RNA content per cell, than the slow-growing K-strategists.

The aim of this study was to test two methods based upon this fact, as possible methods for determining the fraction of r-or K-strategists in seawater communities. One quantitative real-time PCR (rtPCR) method was used to quantify the number of genomic 16S rRNA genes. Maximum cellular RNA content was determined by a flow cytometry analysis of single cells after a nutrient pulse, to induce the cells to grow at maximum specific growth rate. The methods were established in pure cultures and then in intact microbial communities where a shift from K- to r-selection was established, with gradually a succession towards K-selection.

The study in pure bacterial strains confirmed the hypothesis of a correlation between maximum specific growth rate and 16S rRNA gene copy numbers per genome. Moreover, the maximum RNA content of bacterial cells correlated with maximum specific growth rate. In addition, the fast-growing r-strategist was found to divide within 8 hours incubation with nutrients at 15°C, whereas slow-growing K-strategist did not. The r-selected communities showed considerably higher maximum RNA content and higher 16S rRNA gene copy numbers per genome, than K-selected communities.

Of the two methods evaluated, the flow cytometry method was best suited to determine the fraction of r- or K-strategists within seawater microbial communities, e.g. rearing water for marine fish larvae. However, the method is based on a nutrient pulse to achieve maximum cellular RNA content, which is a disadvantage, because the optimal growth conditions for uncultivable cells are not identified. The rtPCR method lacked a precise normalization, and an accurate estimate of the 16S rRNA gene copy number per genome was thus not achieved.

Ideal normalization could be achieved by using a single-copy protein gene and designing universal bacterial PCR primers targeting the gene. If a successful normalization by a single-copy gene is achieved, the rtPCR method, or possibly the proposed digital droplet PCR (ddPCR) method could be alternative methods to determine the fraction of r- or K-strategists in microbial communities. A method like this relies on analysis on DNA level, and would be cultivation independent, as opposed to the flow cytometry method.

Sammendrag

r/K-begrepet er et teoretisk konsept som kan benyttes som verktøy for å evaluere risikoen for innvasjon av patogene bakterielle spesier i oppdrett av marine fiskelarver. I oppdrett av marine fiske larver, kan dominanse av opportunistiske, hurtigvoksende r-strategister føre til lav overlevelse og vekst for fisken. Økt overlevelse og vekst har blitt observert ved dominanse av saktevoksende K-strategister. Generelt har r-strategistene mange kopier av 16S rRNA genet, mens K-strategistene har en eller få. Dessuten, maksimum spesifikk veksthastighet korrelerer også med maksimum RNA innhold i bakterielle celler. Dermed har hurtigvoksende r-strategister et høyere RNA innhold per celle, enn de saktevoksende K-strategistene.

Hensikten med studien var å teste to metoder basert på disse teoriene, som potensielle metoder for å bestemme andel r- eller K-strategister i mikrobielle samfunn i sjøvann. En kvantitativ real-time PCR (rtPCR) metode ble benyttet for å kvantifisere antall 16S rRNA gener per genom. Maksimum RNA innhold ble bestemt med flow cytometri analyse av enkeltceller etter tilsetning av næring, for å indusere cellene til å vokse med maksimum spesifikk veksthastighet. Metodene ble etablert i rene bakterie kulturer og deretter testet i intakte mikrobielle samfunn hvor et skift fra K-til r-seleksjon ble etablert, med gradvis en suksesjon mot K-seleksjon.

Analysen av rene bakterie stammer bekreftet hypotesen om at det er en korrelasjon mellom maksimum spesifikk veksthastighet og antall kopier av 16S rRNA genet. Maksimum RNA innhold i en bakterie celle korrelerer også med maksimum spesifikk veksthastighet. I tillegg ble det observert at de hurtigvoksende r-strategistene hadde celledeling innen 8 timers inkubasjon ved 15°C, mens de saktevoksende K-strategistene hadde det ikke. De r-selekterte mikrobielle samfunnene hadde et betydelig høyere maksimum RNA innhold og høyere antall kopier av 16S rRNA genet, sammenlignet med de K-selekterte samfunnene.

Av de to metodene evaluert, var flow cytometri metoden best egnet til å bestemme andelen av r- og K-strategister i mikrobielle samfunn i sjøvann, for eksempel i vann for oppdrett av marine fiskelarver. Likevel, metoden er basert på tilsetting av medium for å oppnå maksimal spesifikk veksthastighet. Dette er en ulempe, da optimale vekstbetingelser for ukultiverbare celler ikke er identifisert. Real-time PCR metoden manglet en nøyaktig normalisering, og presis estimering av kopiantall av 16S rRNA genet ble dermed ikke oppnådd. Ideell

normalisering kan bli oppnådd ved å benytte et enkeltkopi gen og konstruere universelle bakterie PCR primere for en vellykket amplifisering av genet. Hvis dette oppnås, kan real-time PCR metoden eller den foreslåtte digital droplet PCR metoden, være mulige alternative metoder for å bestemme andelen r- og K-strategister i mikrobielle samfunn. Metodene baserer seg på analyser på DNA nivå og vil dermed være helt kultiverings-uavhengig, i motsetning til flow cytometri metoden.

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1. Introduction

Microbial populations live together in microbial communities, the highest unit in the ecological hierarchy, where they interact with each other (Atlas and Bartha, 1998). Like any other organisms, they strive to survive, maintain and reproduce. To be most successful, they choose superior strategies of growth in different directions. The strategy of growth creates the possibilities to establish methods to enhance knowledge about the composition of microbial communities.

1.1 Microbial ecology

The different members within a community have various tasks, functions, metabolic processes and growth rates. The composition of such a community influences the stability and functionality (Pimm, 1984). The population within a habitat is living in a niche, representing a specialized functional role. There is competition between populations that use the same resources to fill a particular niche and hence play a functional role to maintain the community (Atlas and Bartha, 1998). The abundance (proportion) and the richness (numbers) of species can be used to express the diversity of a microbial community. Both the abundance and the richness can change, and are functions of the community conditions (Madigan, 2012). The resource`s available and environmental conditions like temperature, pH and oxygen content, determine the diversity and richness of the microorganisms in a microbial community. The habitats in the community have different characteristics, which make them favorable for growth of some microorganism, but unfavorable for others (Madigan, 2012).

The stability of an ecological community reflects the effects a perturbation has on the community and the ability to return to its initial state. Common properties that are associated with stability are resilience, resistance, variability and persistence (Pimm, 1984). Resilience is defined as how fast the system can return to its initial state (equilibrium) after a perturbation and therefore, recover. For unstable systems, resilience can thus not be defined. Population densities vary over time and is defined as the variability. When a variable, like species composition or total biomass, is changed to a new value, this reflects the persistence of a microbial community. Resistance is how large the change is due to perturbation of a

community. That is, the ability to withstand disturbance (Pimm, 1984). An increase in both resilience and resistance, will thus lead to improved stability.

The central question that is partly unanswered in ecology is how the biological diversity is connected to ecological function. It's been stated early by ecologists that the diversity influences the stability of a community (MacArthur, 1955, Elton, 1958). The composition of the community and the interactions among the members will have an impact on the stability of that particular community. When a large number of species are present, this can minimize the probability of a large change in the ecosystems function after perturbation (Ives *et al.*, 2000). Thus, the response in diversity and species inventory to variable conditions tends to have stabilizing roles within a community (McCann, 2000). The most fundamental issue in determining the ecological function, is knowledge of the growth and interaction of the populations within a community.

1.2 Population growth

An increase in numbers of cells within a population is per definition microbial growth. A cell division creates two daughter cells from one parental cell and the time it takes is referred to as the generation time. The generation time varies widely among microorganisms and depends on genetics and different environmental and nutritional factors. Some rapidly growing bacteria have generation times of under 0.2 hours during the optimal growth conditions, whereas some slow-growing bacteria have been shown to have generation times of several days or weeks (Madigan, 2012).

Temperature and nutrient availability are factors that influence the exponential growth of a population. In addition, the genetic and metabolic capacity of the organism itself influence the growth. Small cells, for example, have higher capacity for nutrient exchange than larger cells, due to a high surface to volume ratio (S/V ratio), and this can affect the growth rate in a positive direction (Madigan, 2012). Interactions between the microbial populations in a habitat also affect the growth, e.g. by the production of growth factors (Bell *et al.*, 1974).

In natural environments, long term periods of exponential growth are rare. The growth rates are usually well below the maximum growth rate, due to the availability and nature of

resources. It has been indicated that soil-bacteria in the nature grow at rates less than 1% of the maximum growth rates that have been recorded by cultivation in the laboratory (Madigan, 2012). This can be explained by suboptimal and not equally distributed nutrients in the habitats. In a habitat, there is a mixture of different species, which compete strongly for resources. The overall growth of a population is dependent on cell division and death rate within the population (McArthur, 2006). To achieve the highest growth rate and density in a habitat, the population must be in its prime niche, where it's most successful. The population may also inhabit other niches, where they will be able to compete, but with less ecological success (Madigan, 2012).

An important point in microbial ecology is to increase the understanding of how different members of a community interact and influence each other's behavior. Paramount theories have been formed to simplify this work, like the theory of r- and K-selection.

1.3 The theory of r- and K-selection

r/K selection is a theory of how an organism optimizes its fitness within a community. The basis of the theory is the logistic equation of growth, which the r and the K terms have arisen from (McArthur, 2006). The logistic equation of growth is defined as: $dN/dt = rN((K-N/K)$ (McArthur, 2006), where r is the rate of increase of a population, N is population size and K is the carrying capacity. The carrying capacity is the maximum number of individual (maximum biomass) that an environment can support. This can be influenced by a set of factors and the interactions between them. Availability of nutrients, space, temperature, water and other factors is influencing K (McArthur, 2006).

The r/K-selection theory was first published by MacArthur and Wilson (1967), a publication based upon their work on Island Biography. They studied the ecology of colonizing species in different stages, and suggested that this colonization could be defined as r- or K-selection. The r/K-strategy essence is that the organism optimizes their fitness in uncrowded or crowded environment, and that a high r leads to a low K and vice versa (Andrews and Harris, 1986, Wilson and Bossert, 1971).

Uncrowded environments with a low population density provide r-selection (r-strategists). Environments like this are often unstable and unpredictable, and the microorganisms that have a high maximum growth rate (high r), will be favored. Crowded environments with a high population density, on the other hand, provide for K-selection (K-strategists) (Andrews and Harris, 1986). r-strategists are opportunistic and do not compete well for nutrients. In addition, pathogenic species are in general characterized as r-strategists (Andrews, 1984). The r-strategists are dependent on a fast reproduction rate for survival within a community. Typically, they are the initial colonizers in a habitat and will dominate in such a pioneer community, because the competition for resources is low and nutrient availability tends to be high (Atlas and Bartha, 1998). A pioneer community will generally have low stability against perturbations, and a low biological control is achieved (Vadstein *et al.*, 1993). The term biological control is based upon the phenomenon where pathogens can be killed or reduced in number, by the natural competition among microbes in the aquatic environment (Maeda *et al.*, 1997).

The K strategist takes over the community when the environment stabilizes, and nutrient levels are reduced. K-strategists have a slower maximum growth rate than r-strategists, as they use less of the resources on reproduction. They are thus most successful in resource limiting environment and exploit lower nutrient concentrations better than the r-strategists. Because of that, the K-strategists are often more permanent members of a community (Atlas and Bartha, 1998). Such a mature community will have a high resistance to perturbation and the diversity and biological control is high (Vadstein *et al.*, 1993).

The overview of the characteristics of the r- and K-strategist, as well as the characteristics of pioneer- and mature communities are given in Table 1.1.

Table 1.1: The characteristics of pioneer- and mature microbial communities in relation to r- and K-strategists. Data from Vadstein *et al.* (1993).

Community level	**Pioneer community**	**Mature community**
Biological control	Low	High
Stability to perturbation	Poor	Good
Diversity	Low	High
Specialization	Low	High
Species level	**r-strategist**	**K-strategist**
Maximum growth rate	High	Low
Effect of enrichment	Rapid growth	Slow growth
Affinity to substrate	Low	High
Competitive ability; low nutrient supply per capita	Poor	Good

The quantitative extreme, the r-endpoint, will have no competition and all energy is put into reproduction. The qualitative extreme represented by K-endpoint, will have the maximum density, with a saturated environment. The competition is complete and all energy is used for maintenance (Pianka, 1970). The theory of r- and K-selection depends on these two extremes, and it is important to remember that some organisms may be somewhere in between (McArthur, 2006).

Many studies over the year on microbial diversity, has created experimental evidence for r/K-selection also among microorganisms (Veldkamp *et al.*, 1984, Andrews and Harris, 1986). Especially the relationship between nutrient concentration and specific growth rate and its influence in success has been the main focus in these studies. To determine the degree of r-or K-selection in microbial communities, the first step is to characterize the different members within the community.

1.4 Characterization of microorganisms and methods to determine the degree of r/K-selection in mixed microbial communities

Cultivation of microorganisms has been a common method to isolate and characterize members of microbial communities. In standard cultivation methods, the organisms are isolated by their ability to grow on media with high nutrient concentrations and form colonies (Hugenholtz, 2002). It is a broad agreement that the easily cultivable organisms do not represent the majority of the microbial world. The "great plate count anomaly" is due to the discrepancy between the viable count by plating and total counting by microscopy (Staley and Konopka, 1985). Staley and Konopka (1985) stated that 0.1 to 1% of heterotrophic bacteria in oligotrophic and mesotrophic aquatic habitats are cultivable. In eutrophic habitats, the percentage is found to be higher, around 80% (Staley *et al.*, 1982).

Uncultivable organisms were for a long time a challenge to study. Due to molecular methods, new knowledge about microbial diversity and ecology has arisen. We are now able to analyze also uncultivated microbial communities, by the use of culture-independent methods (Madigan, 2012). Genomics is referred to as mapping, analyzing, sequencing and comparisons of functional genes. Especially the gene encoding 16S rRNA has been important in genomics within the microbial ecology field (Madigan, 2012). By amplification of 16S rDNA (fragment of the 16S rRNA gene) by PCR (polymerase chain reaction), there is no need for cultivation and fragments of the gene can be sequenced and compared among different strains (McArthur, 2006). The 16S rRNA has become the standard marker gene for studying evolutionary history and phylogenetic relationship, diversity, community structure/dynamics between bacteria today. It is found in all procaryotic organisms and is useful as a marker because the gene is little affected by horizontal gene transfer (Atlas and Bartha, 1998). The changes in these sequences over time through generations are thus evolutionary. In addition, the 16S rRNA gene contains hypervariable and highly conserved regions. The hypervariable regions are used to determine the phylogenetic relationship, because the region differs greatly between related organisms. The conserved regions are similar in all organisms and are therefore an excellent target of universal PCR primers (Kirchman, 2012). The 16S rRNA sequence has not been rapidly changed due to selection, because it has a central function in protein synthesis (Atlas and Bartha, 1998).

New species have been discovered by sequencing, species that are uncultivable and thus previously unidentified (Madigan, 2012). Such molecular methods have provided important information regarding the composition of microbial communities. Consequently, it has become relatively easy to determine who the members of a microbial community are due to the new molecular methods. The obstacle is to define how the different members interact and grow, and how this affects the structure and function of a community.

To estimate the fraction of r-strategic bacteria in a community today, we only have cultivation dependent methods. Salvesen and Vadstein (2000) based their method on counting of fast-growing bacterial colonies from seawater on agar plates to determine the frequency distribution of maximum specific growth rates in mixed communities. Similar cultivation methods have also been tested in mixed microbial communities in soil and roots and in rearing systems for marine fish larvae (De Leij *et al.*, 1994, Salvesen *et al.*, 1999). However, as normally about 99% of the microbial cells present in the environment are uncultivable, cultivation based methods could be biased. The drawback of culture-dependent methods are thus the uncertainty that the cultivable cell represents the community as a whole. In addition, cultivation based methods are time consuming due to the need of cultivation.

1.5 Correlation between specific growth rate and RNA content

The information that is stored in the DNA is being transferred to ribonucleic acid (RNA) when the genes are expressed. Three different classes of RNA are involved in protein synthesis: Messenger RNA (mRNA), ribosomal RNA (rRNA) and transfer RNA (tRNA). The rRNA is an important catalytic component in the ribosomes, as well as a structural unit (Madigan, 2012). The content of RNA is high in bacterial cells. In *Escherichia coli* strain B grown on a minimal medium, RNA stands for 1/5 of the dry mass of the cell. Approximately 80% of the RNA is rRNA (Neidhardt *et al.*, 1990).

It's been documented that an increase in the size and the RNA content of a cell is well connected (Morse and Carter, 1949, Gale and Folkes, 1953). The macromolecular composition of RNA in a cell, and its relation to the metabolic activities to protein synthesis, were also demonstrated by Schaechter *et al.* (1958). They cultivated *Salmonella typhicurium* with different growth media and temperatures, discovering that at a given temperature, the

amount of DNA, RNA and proteins in a cell are exponential function of growth rate. In addition, they saw that fast-growing bacteria (r-strategists) are larger and contains more of these macromolecules, than the slow-growing bacteria (K-strategists). This supports the results of Morse and Carter (1949), and Gale and Folkes (1953).

Due to a greater requirement of protein synthesis when the growth rate increases, the amount of RNA and therefore, the number of ribosome`s also increases. As the cell grows bigger, it contains more of its components and this change with the growth rate. The amount of each component is thus predictable according to the growth rate (Schaechter *et al.*, 1958). An example is that an *Escherichia coli* B strain that doubles 0.6 times per hour contained 10 times less RNA than a culture growing with 2.5 doublings per hour. The fraction of each of the three components was a linear function of growth rate, as long as the doubling was over 0.5 per hour (Neidhardt *et al.*, 1990). The relationship between growth rate and the relative amounts of RNA, mass, protein and DNA is shown in Figure 1.1.

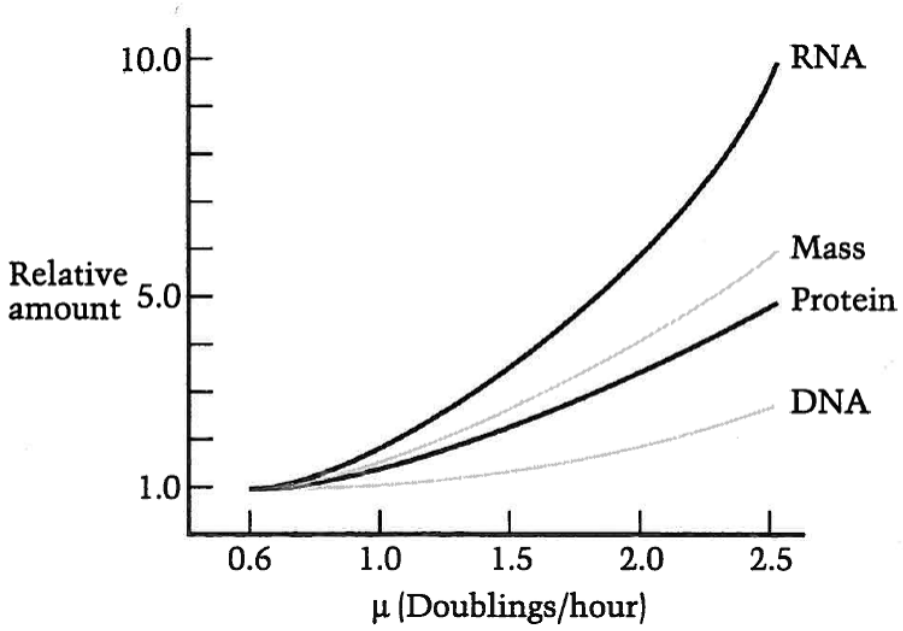

Figure 1.1: After nutrient imposed growth, the relative amount of RNA, mass, protein and DNA is given as a function of growth (μ). Collected from Neidhardt *et al.* (1990). Plotted from data in Bremer and Dennis (1987).

On the background of the macromolecular composition of bacterial cells, it is expected that the fast-growing r-strategists will have a higher maximum RNA content in their cells, compared to the slow-growing K-strategists.

1.6 Correlation between 16S rRNA gene copy numbers and maximum specific growth rate

Ribosomal RNA (rRNA) is the structural component of the ribosomes and possesses a catalytic function as mentioned in the previous section. It is produced by transcription of the gene encoding it, but no translation is involved. The rRNA is thus the final gene product, and the rRNA gene does not encode polypeptides like mRNA (Snustad and Simmons, 2012).

The ribosomes consist of protein and rRNA, about half and half of each. The small (SSU) and the large subunit (LSU) contain a folded rRNA molecule each with assembly of proteins on them. The 30S small ribosomal subunit contains the 16S ribosomal RNA (Figure 1.2). The size of the ribosomes is expressed in Svedberg (S) units: rate of sedimentation during centrifugation (Snustad and Simmons, 2012). Composition of the ribosomes is illustrated in Figure 1.2.

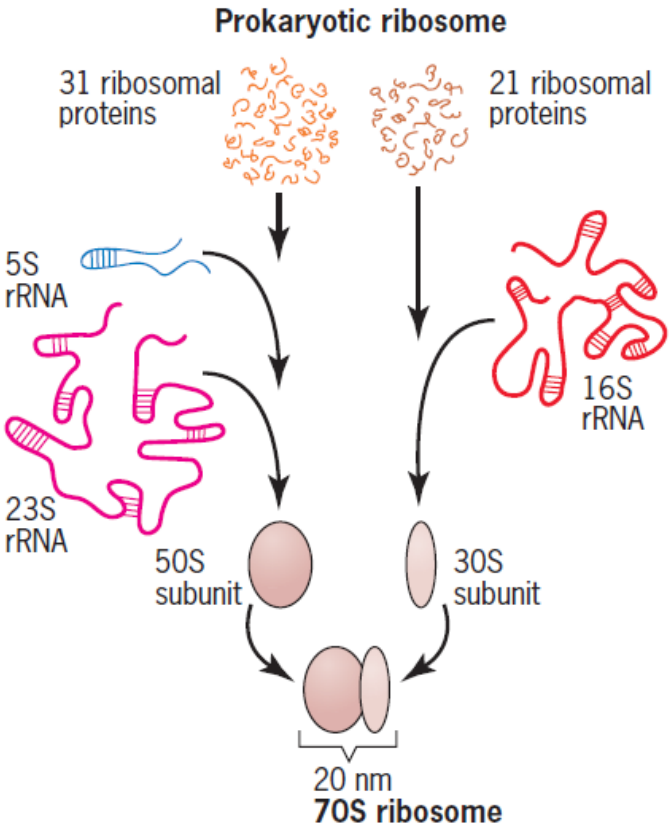

Figure 1.2: Composition of the prokaryotic ribosome. The 16S forms the 30S (SSU) subunit in association ribosomal proteins. 5S, 23S and ribosomal proteins form the 50S (SSU) subunit. The two subunits associate to form the 70S ribosome when the translation of mRNA is initiated (Snustad and Simmons, 2012).

rRNA function as information processors and because of that fact, they probably established early in the common ancestors of all organisms; Bacteria, Archaea and Eucarya. In species among the domain Bacteria, genes encoding 5S, 16S and 23S are organized into operons. Different species contain unequal copy numbers of the rRNA operon and it varies between 1 and 15 copies (Stoddard and Smith, 2015). It's generally assumed that the copy number of rRNA operons in bacteria is related to maximum growth rate. From a rRNA operon promoter, a certain number of rRNA transcripts can be initiated. This, in addition to the RNA polymerase activity, sets a maximum number of ribosomes that is being produced from one single operon per unit of time (Klappenbach *et al.*, 2000). From an estimate of promoter initiation and transcription rates, it has been calculated that the high maximum specific growth rate observed in *Escherichia coli* requires more than one copy of the rRNA operon (Bremer, 1975). This fits reality, as the 16S rRNA gene copy number of *E.coli* is 6 or 7 in sequenced strands (Stoddard and Smith, 2015).

Klappenbach *et al.* (2000) showed that there is a correlation between the 16S rRNA gene copy number in soil bacteria and the rate of response to a variety of growth substrates. Their results indicated that multiple rRNA genes are connected to the competitive ability of bacterial species, and that it can provide a genetic indicator of bacterial strategies for exploitation of nutrients. Vieira-Silva and Rocha (2010) also found a correlation between the number of 16S rRNA genes and growth rate (Figure 1.3). This assumption is now commonly accepted.

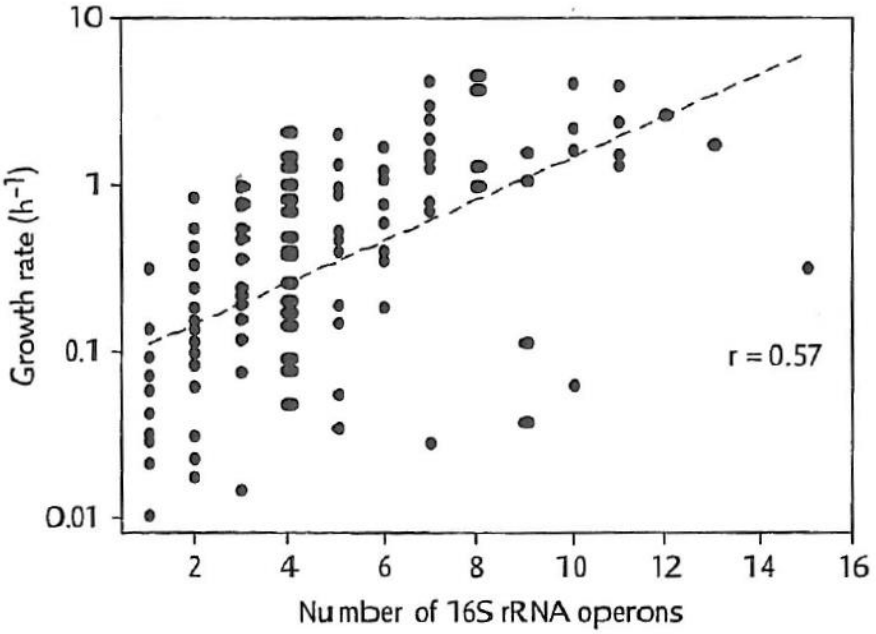

Figure 1.3: Correlation between 16S rRNA gene copy number and log growth rate. Data from Vieira-Silva and Rocha (2010). Copied from Kirchman (2012).

On this background, it can be concluded that high copy numbers of the rRNA operon support a high maximum growth rate of an organism. Organisms with a high growth rate, i.e. r-strategists, in general have high copy number of the 16S rRNA gene. K-strategists on the other hand, have one or a few.

1.7 Application of the r/K concept

Eggs and newly hatched fish larvae become colonized, and the colonization of the gastrointestinal tract (GI) is important for fish development and health (Nayak, 2010). This colonization and establishment of microbiota, is thought to be a reflection of the microbial composition of the rearing water, the diet and their environment (Nayak, 2010, Korsnes *et al.*, 2006, Geldreich and Clarke, 1966, Ringø *et al.*, 2006). In zebrafish the epithelial surface is colonized at birth by large communities of microorganisms that form mutualistic or commensal relationship with their host (Cheesman and Guillemin, 2007). These microbial cells may influence the immune cell production of the zebra fish (Kanther and Rawls, 2010).

In aquaculture, the larvae are exposed to a different microbial environment than in the nature. Large variation of viability in the rearing of marine fish larvae is a major problem and most likely caused by other factors than nutrition and egg quality (Vadstein *et al.*, 1993). It has been suggested that this poor survival is connected with the proliferation of opportunistic pathogenic bacteria (Nicolas *et al.*, 1989, Muroga *et al.*, 1987). A combination of nutrient supply from the live food and the poorly developed immune system provides good conditions for the detrimental r-selected species (Vadstein *et al.*, 2004).

The influence of the microbial flora in the environment with respect to primary colonization is not fully understood. In theory, if the primary colonizers consist of non-opportunistic bacteria (K-strategist), this would be beneficial to the larvae, because the K-strategist could protect the larvae from colonization by opportunistic bacteria (Skjermo *et al.*, 1997). Pathogens are in general opportunistic r-strategists, but not all r-strategist are pathogens. The problems with rearing of larvae may also be caused by non-pathogenic r-strategists (Vadstein *et al.*, 1993). When feeding the larvae, a high load of organic matter and bacteria is supplied to the system. This selects for opportunistic r-selected species that can be potentially harmful to the fish (Vadstein *et al.*, 1993) To stabilize the system by reducing the organic input and select for non-opportunistic bacteria (K-strategists), increased survival and growth of marine larvae has been seen (Skjermo *et al.*, 1997, Vadstein *et al.*, 1993, Skjermo and Vadstein, 1999).

The successful application of the r/K concept for understanding fish-microbe interactions in aquaculture points to the need for establishing a culture-independent method for characterizing microbial communities on a r/K axis.

1.8 Aim

Today we only have cultivation-dependent methods to estimate the fraction of r-strategic bacteria in a community (Salvesen and Vadstein, 2000). The challenge is that such methods are time consuming and unreliable as most bacteria cannot be cultivated. It is therefore a need to establish cultivation-independent methods. A method like this would be a valuable tool for evaluating the structure of microbial communities and microbial quality of rearing water in aquaculture.

The overall goal of this master thesis was to establish two different cultivation-independent methods to determine the fraction of r-strategists in microbial communities:

- One qualitative PCR based method to quantify the average 16S rRNA gene copy number per genome.
- One Flow cytometry based method to quantify the maximum RNA content in single cells.

First, the hypothesis was tested in pure bacterial strains with variable maximum specific growth rate that represented r- and K-strategists:

- Maximum RNA content correlates with maximum specific growth rate (μ_{max}). Fast-growing r-strategists have a higher maximum RNA content per cell, compared to the slow-growing K-strategists.
- Fast-growing r-strategists with a high maximum specific growth rate have a high genomic copy number of the 16S rRNA gene, compared to the slow-growing K-strategists, who has one or a few copies of the gene.

Further, the methods were tested in mixed microbial communities where a shift from K-to r-selection, and a gradual succession back to K-selection was established. Assessment was implemented according to the study overall aim and to what degree it was possible to determine the fraction of r- or K-strategist by means of the two methods.

2. Material and methods

2.1 Principle of some methods used

2.1.1 Flow cytometry

Flow cytometry is a useful tool for analyzing and characterizing single cells, including microbial cells. It supports high throughput and rapid processing of multiple samples and gives information regarding the number of cells, size and biological- and metabolic properties of individual cells. It has been well established in microbial studies of aquatic samples (Díaz *et al.*, 2010, Wang *et al.*, 2010). A wide range of fluorescent dyes are available to characterize different features of bacterial cells, like DNA, RNA, viability and granularity, making flow cytometry a valuable tool for microbial ecology studies (Wang *et al.*, 2010, Gasol and Del Giorgio, 2000, Lebaron *et al.*, 1998).

The flow cytometer consist of three main systems (BD Bioscience, 2000):

- Fluidics: Transports the particles to the laser beam in a narrow stream.
- Optics: Lasers that illuminate the particles and filters that direct the light signals to the detectors.
- Electronics: Convert the detected light signals into electrical signals that can be processed by a computer.

When a cell suspension is drawn into the instrument, an isotonic fluid creates a laminar flow, so that the cells in the suspension align in a narrow stream. The single cells pass a powerful light source, often a laser, and scatter of light occurs each time a particle passes. The size, shape and refractive index determine the angular intensity (Marie *et al.*, 2005). If compounds of the cells is stained with a fluorescent label that has an absorption spectrum that corresponds to the laser (excitation source), the laser excites the fluorescent molecules to a higher energy state (Marie *et al.*, 2005). The fluorochromes emit light at longer wavelengths, when returning to resting state (Brown and Wittwer, 2000).

The light scattering that occurs when a particle deflects incident laser light, depends on the size and internal complexity of the particle. Cell shape, nucleus, cell membrane and granular material inside cells will affect the light scattering. Forward-scattered (FSC) light is

proportional to the size or the cell-surface area. Side-scattered (SSC) light reflects the internal complexity of the cell and is a measurement of refracted and reflected light (BD Bioscience, 2000). The emitted light that is given off in all directions is collected by optics and directed to filters. The filters in combination with dichroic mirrors send the light signals into detectors that collect light at particular wavelengths (Brown and Wittwer, 2000). Components and configuration is illustrated in Figure 2.1.

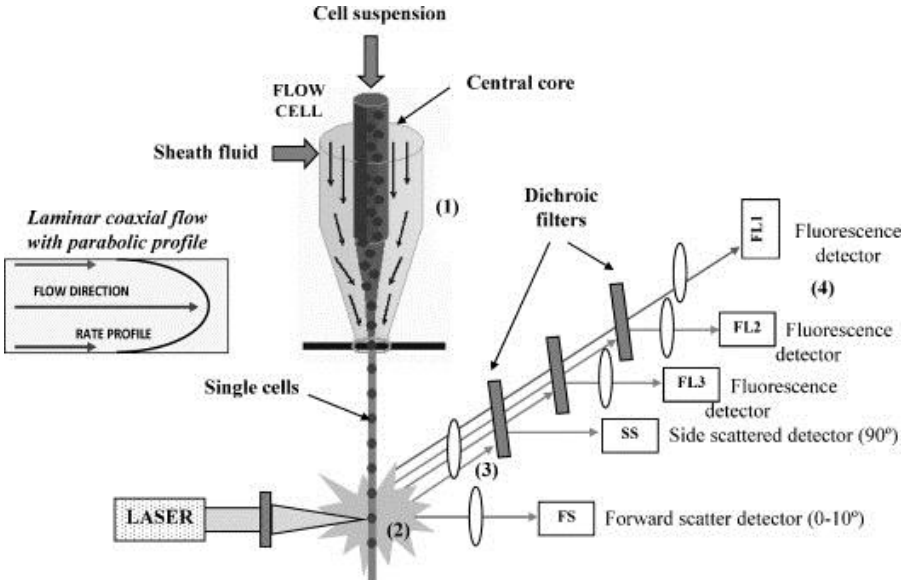

Figure 2.1: Schematic overview of the components of a flow cytometer. Cell suspension is drawn through the flow cell and single cells are aligned. The laser light creates emitted light that is collected in the different detectors. Figure from Díaz *et al.* (2010).

2.1.2 Quantitative PCR

2.1.2.1 Real-time quantitative PCR

Polymerase chain reaction (PCR) is a powerful technology used for quantification of nucleic acid. In real-time quantitative PCR (rtPCR), the PCR product is measured at each cycle of the reaction, due to labeling of the amplified DNA by DNA binding dyes. The dyes are either double-stranded DNA (dsDNA) binding dyes, or dyes that are attached to primers/probes. DNA binding dyes bind to all products that are double stranded, whereas primer/probes give sequence specific marking. The dyes hybridize with the PCR amplicons that are generated, and a fluorescent signal occurs. The fluorescence is proportional to the amount of PCR

amplicons generated. By collecting data in the exponential phase of the reaction, the starting concentration of the target gene can be determined (Thermo Fischer Scientific, 2014).

The "Cycle threshold" (Ct) is identified as the cycle where the "Threshold line" is met (Figure 2.2). Fluorescent signal below the "Threshold line" is considered background noise, and thus the threshold has to be evaluated in the reaction. If the threshold is too low, background noise can influence the samples. If too high, on the other hand, the reaction may not be in its exponential phase and lead to incorrect data collection (Fraga *et al.*, 2008).

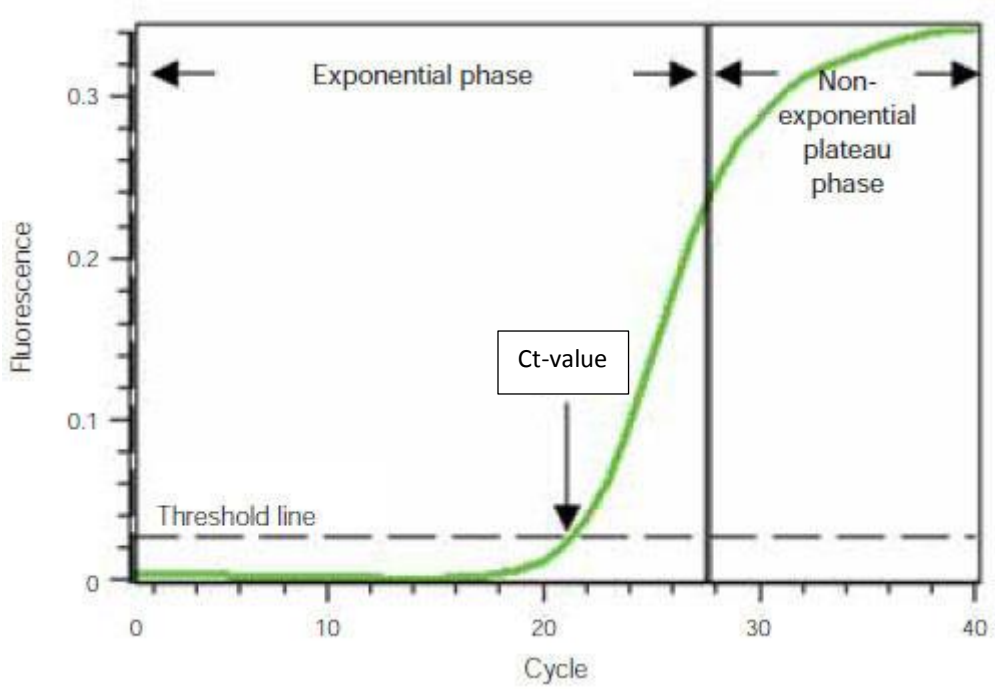

Figure 2.2: The threshold line, the exponential and non-exponential phase of the PCR reaction. The Ct value is the cycle number where the fluorescent signal crosses the threshold line (Bio-Rad Laboratories, 2006).

In the exponential phase of the PCR reaction, a doubling of the product at each cycle occurs, provided that the amplification efficiency is 100% (Fraga *et al.*, 2008) An amplification efficiency of 100% is not always achieved. To compare samples, it is thus important to calculate amplification efficiency obtained from a given primer pair. The amplification efficiency obtained from a primer pair is dependent on the primer binding sites and the length of the target, in addition to the sequence of the amplicon (Fraga *et al.*, 2008).

Real-time PCR has many advantages compared to end-point PCR: 1) Monitoring the process of the reaction, 2) Amplicons at each cycle are recorded and this makes it possible to quantify

the starting concentration of the target gene, 3) Amplification and detection of target gene in the same reaction, so it is no need for post-PCR analysis, such as gel electrophoresis (Thermo Fischer Scientific, 2014).

2.1.2.2 Digital droplet PCR

Digital droplet PCR (ddPCR) relies on water-in-oil generated droplets on a microfluidic chip (Beer *et al.*, 2007, Beer *et al.*, 2008, Kiss *et al.*, 2008). From a reaction volume of 20 µl, ~20 000 droplets can be achieved (Hindson *et al.*, 2011). The generated droplets will contain one, a few or none copies of the template DNA. A traditional end-point PCR is run on the droplets to amplify the target, and the amplified droplets are transferred to a droplet reader. Detection of the droplet is fluorescent based and the signal of the droplets containing the target is detected. A threshold value determines whether a droplet is positive or negative. Due to the random distribution of the template in the droplets, the data fit a Poisson distribution that can be used to calculate the original copy number in the sample (Hindson *et al.*, 2011, Pinheiro *et al.*, 2011).

The advantages of ddPCR seem to be many compared to its sibling, real-time PCR (rtPCR). There are no requirements of a calibration curve and reliance on Ct values (Hayden *et al.*, 2013). The rtPCR the quantification is dependent on amplification efficiency. The quantification with ddPCR is not, as it is based an end-point PCR detection (Sze *et al.*, 2014). As long as the fluorescence intensity is higher in positive droplets compared to the negative one, the quantity of the target gene can be calculated with a high degree of confidence (Sze *et al.*, 2014). When detecting a low copy number in nucleic acid, previous studies have shown a high degree of sensitivity and precision in ddPCR compared to rtPCR (Sanders *et al.*, 2011, Hindson *et al.*, 2011).

2.2 Experimental design

The overall aim of the project was to evaluate whether the two methods, flow cytometry and real-time PCR (rtPCR), were accurate and reliable for determining the fraction of r-strategists in seawater communities.

To investigate if RNA content correlates with the maximum specific growth rate (μ_{max}), pure bacterial strains were isolated from seawater by plating. The growth rate was evaluated by recording the time needed for the appearance of visible colonies through a time period of 14 days.

The isolated colonies were cultivated additionally and sampled in the exponential phase of growth to determine maximum RNA content and in stationary phase (no growth) to determine minimum RNA content with flow cytometry. Furthermore, to examine if the genomic 16S rRNA gene copy number is correlated with μmax, DNA was isolated from the pure cultures and the relative 16S rRNA gene copy number determined by real-time PCR. For the pure cultures, a region of the 16S rRNA gene was also sequenced for taxonomic classification, and to compare the 16S rRNA gene copy number with previously reported findings.

First, the methods were established for the pure strains, and then they were applied in mixed microbial communities. The maximum cellular RNA content and the 16S rRNA gene copy number were determined for mixed microbial communities where a shift from K-to r-selection was established. This was carried out by adding nutrients to two different sea water communities and then sampling them over a time period of 25 days. The hypothesis was that the nutrient added would create an r-selected community. After a period of time the nutrients become limiting and a gradual succession towards a K-selection would occur. By determining the maximum cellular RNA content by flow cytometry and the genomic 16S rRNA gene copy number by real-time PCR, comparison of r-selected and K-selected communities could be conducted.

2.3 Biological material

2.3.1 Seawater with mixed microbial communities

Two seawater types with intact mixed microbial communities were used in this project. This included surface seawater from the Trondheimsfjord (Brattøra) and seawater collected from 90 meters depth in the Trondheimsfjord (received from NTNU Sealab). The application of the seawater is described under each experiment.

2.3.2 Isolation of pure bacterial strains

M65 agar (Slaatebræk, 1975) was used for cultivation of surface seawater from the Tronsheimsfjord to isolate pure bacterial strains. The agar was prepared by mixing 0.5 g/l peptone; 0.5 g/l tryptone; 0.5 g/l yeast extract; 200 ml distilled water; 800 ml filtrated seawater and 15 g/l agar (Appendix A). It was autoclaved in 20 minutes at 120°C before distributed on Petri dishes. To solidify, the agar plates were left at room temperature in 24 hours and then put in plastic bags to prevent them from drying out. They were afterwards kept in a refrigerator.

In seawater, the microbes are expected to compete for nutrients, resulting in K-selection. In theory, the number of fast-growing r-strategist will be low compared to the slow-growing K-strategists. For cultivation of mainly K-strategist, undiluted surface seawater and tenfold dilution series ($1:10^1$ to $1:10^3$) were plated on M65 agar plates. The dilutions were done with M65 liquid medium with a concentration of 0.5 g/l of each component (Appendix A). To create a selective shift-up and select for the r-strategist in the seawater to grow, M65 stock medium (150 g/l) (Appendix A) was added to a final concentration of 150 µg/m (in total of the three components). The surface seawater community was incubated at 15°C (Termaks, Bergen, Norway) in two days, and then plated out on M65 agar. Tenfold dilution series were made ($1:10^1$ to $1:10^5$), as it's expected higher bacterial counts subsequent to nutrient addition. Agar plates were incubated at 15°C (Termaks, Bergen, Norway) for 12 days.

The colony-forming units (CFU) were counted each day at the same time and hours for the formation of a visible colony was registered. This time determines 1/T, which is equal to maximum specific growth rate (μ_{max}) (Salvesen and Vadstein, 2000).

2.3.3 ATCC reference strains

Two cell lines, *Bacillus licheniformis* (ATCC®14580™) and *Hyphomonas neptunium* (ATCC®15444™) were acquired from ATCC (Manassas, USA) and used as reference strains for copy number determination of the 16S rRNA gene. The 16S rRNA gene copy number for these strains were known from The Ribosomal RNA Database (Stoddard and Smith, 2015). The strains were delivered as freeze dried and cultivation in the appropriate mediums was required to isolate DNA.

H.neptunium was cultivated aerobically on Difco ™ Marine agar (BD Biosciences, San Jose) and in Difco ™ Marine broth (BD Biosciences, San Jose) in Minitron HT (Infors AG, Bottmingen) in 26°C at 150 rpm (revolutions per minute) for 48 hours. This strain has one copy of the 16S rRNA gene (Stoddard and Smith, 2015). *B.licheniformis* were cultivated aerobic on Difco™ Nutrient agar (BD Biosciences, San Jose) and in Difco™ Nutrient broth (BD Biosciences, San Jose) at 37°C at 225 rpm for 24 hours. The genomic 16S rRNA gene copy number of the strain is 7 (Stoddard and Smith, 2015). Rpm of both cell lines was chosen for practical reasons.

To ensure pure cultures, one colony of each was picked from agar after cultivation with disposable sterile inoculating loops and inoculated in a cultivation tube (Sahrstedt AG &Co, Nümbrecht) containing the respective broth mediums. The incubation time and temperature performed as previous, with sampling for DNA isolation at the end of the incubation period (section 2.5.2.1).

2.4 Experiments

2.4.1 Effect of temperature and incubation time on RNA content and cell division

To identify the temperature and incubation time needed for a microbial community in seawater to obtain maximum cellular RNA content, without cell division of any of the members, surface seawater from the Trondheimsfjord (Brattøra) was used.

The surface seawater was sampled directly in two sterile disposable 400 ml cultivation flasks (Thermo Fisher Scientific Inc., Waltham). M65 stock medium (150g/l) was added to the seawater to a final concentration 150 µg/ml. 40 µl f/2 medium (Guillard, 1975) (total concentration of components in Appendix B) was also added. In addition, NaH_2PO_4 was added to a total concentration of 0.5 µg/ml. The two flasks were incubated in 12+0.3°C (Minitron shaker, Infors AG, Bottmingen) and 22±0.5°C, respectively. Sampling for determination of cell density and RNA content by flow cytometry was done before adding the medium, after 1, 2, 4, 8, 12 and 24 hours (section 2.5.1.1).

2.4.2 Cultivation of pure strains representing r- and K-strategists to sample for minimum/maximum RNA content and 16S rRNA gene copy number determination.

To determine maximum and minimum RNA content of pure strains representing r- and K-strategists and to evaluate whether the maximal cellular RNA content and 16S rRNA gene copy number is correlated to μ_{max}, the pure strains were cultivated and sampled in the exponential and stationary phase of growth (section 2.3.2).

The colonies visible at various times (μ_{max}) from the isolation of the pure cultures were picked with disposable inoculating loops, inoculated in approximately 10 ml M65 medium (500 mg/l) in glass tubes, and incubated at 15°C (Termaks, Bergen) for 8 hours. After 8 hours, incubation at 21°C for a more rapid growth to reach stationary phase was induced. To be able to reanalyze the cultures, freezing stocks of the samples with a final concentration of 20% glycerol was made in cryotubes. After addition of glycerol, the stocks were frozen in -80°C. The experiment was also repeated in Erlendmeyer flasks, as the fast growing r-strategist had

insufficient oxygen supply in the glass tubes. To get enough oxygenation, incubation was performed in Minitron shaker (Infors AG, Bottmingen, Switzerland) at 15°C and 90 rpm.

Sampling for determination of maximum RNA content by flow cytometry during in the exponential phase (μ_{max}) was done after both 4 and 8 hours. As a proxy for biomass, the absorbance of the cultures were measured daily with U-5100 spectrophotometer (Hitatchi, Ltd., Tokyo, Japan), to determine when the cultures were in the stationary phase, and no growth was expected. When there was no increase in absorbance, the cultures were sampled for flow cytometry to determine minimum RNA content (section 2.5.1.1). At the same time, sampling for DNA isolation for determination of the 16S rRNA gene copy number and sequencing was performed (section 2.5.2.1).

2.4.3 Cultivation of r- and K-selected communities to sample for determination of minimum/maximum RNA content and 16S rRNA gene copy number

To obtain samples for determination of the average 16S rRNA gene copy number per genome and the maximum RNA content of single cells in mixed microbial communities, a shift from K-to r-selection and a gradual succession to K-selection was implemented in two different microbial communities.

Surface seawater from the Trondheimsfjord (Brattøra) with an intact microbial community was collected. Further, seawater collected from 90 meters depth in the Trondheimsfjord was received from NTNU Sealab. It was an assumption that the two seawater types would have different microbial compositions, and that the 90 meter water had a lower carrying capacity (more K-selected) than the surface water, due to lower nutrient availability in the deep.

Two liters of each water sample were collected in autoclaved Nalgene flasks (Thermo Fisher Scientific Inc., Waltham). To trigger r-selection, M65 stock medium (150 g/l) was added to both flasks to a final concentration of 25 µg/ml. This one time addition of medium is further referred to as initial shift. The seawater was incubated at 15°C in an incubator (Termaks, Bergen) for 24 days and mixed at 300 rpm with MR3001 magnetic stirrer (Heidolph, Schwabach) the entire incubation time. To prevent algae growth, the samples were incubated in the dark.

The microbial communities were sampled during a time period of 24 days. The expectations was K-selection at day 0, shift to r-selection at day 1-2 and gradually succession to K-selection towards day 24. Sampling was performed at day 0 (before adding the medium), day 1-5, day 7, day 9, day 13, day 17, day 20 and day 24. Sampling included:

- Approximately 50 ml for DNA isolation to determine the average genomic 16S rRNA gene copy number.
- Approximately 4 ml for flow cytometry to quantify cell densities and RNA content (unpulsed sample).
- 25 ml to incubate with a new addition of the medium (pulsing) to trigger maximum RNA content of the members in the community (see below).

The purpose of the incubation with additional medium (pulsing) was to trigger a maximum RNA content of all the members in the communities and determine cell densities compared to the unpulsed sample on each day. Exactly 25 ml was aseptically taken out of the primary cultivation flask with a disposable sterile pipette and transferred into 40 ml cultivation flasks (Thermo Fisher Scientific Inc., Waltham, USA). M65 stock medium (150 g/l) was added to a final concentration of 25 µg/ml. Samples were incubated for 6 hours at 15°C in an incubator (Termaks, Bergen, Norway) in the dark, just like the primary cultivation flasks. At exactly 6 hours of incubation, 1-2 ml was sampled for flow cytometry to quantify cell densities and RNA content of single cells. For the performance of sampling, see section 2.5.1.1 and section 2.5.2.1.

2.5 Analytical methods

2.5.1 Flow cytometry

2.5.1.1 Sampling for flow cytometry

Sampling for determination of maximum and minimum RNA content was done aseptically in a laminar flow cabinet with a disposable pipette, and samples were distributed into cryotubes (VWR International, Radnor). To fixate the samples and stop further metabolism, glutaraldehyde (50%) were added to a final concentration of 0.1%. The samples were mixed with an MS2 minishaker (IKA®-Werke GmbH & Co.KG, Staufen) and snap frozen in liquid nitrogen. Samples were stored at -20°C until analysis.

2.5.1.2 Staining of RNA

Samples were taken out of -20°C freezer and thawed at room temperature. To homogenize the samples, they were mixed well with Paramix 3 vortexer (Julabo, Seelbach). The maximum cell number to be counted on the flow cytometer is 1 million counts per sample run (BD Bioscience, 2012). Samples were diluted in 0.2 µm filtrated seawater from 1:2 to 1:200, dependent on cell densities.

SYBR®Green II RNA Gel Stain (Life Technologies, Thermo Fisher Scientific Inc., Waltham,) was used for staining. This fluorescent stain binds to RNA in bacterial cells and is useful for enumeration of cells in aquatic systems (Lebaron *et al.,* 1998). The stain is maximal exited at 497 nm (Appendix C). The emission of the dye is centered at 520 nm. The SYBR®Green II RNA Gel Stain were provided as a 10 000X concentration. A working solution (1:100) was prepared from the stock solution on the day of analysis. 10 µl/ml working solution was added to the sample in a total volume of 1 ml, mixed and incubated in the dark at room temperature for 15 minutes before analysis.

2.5.1.3 Analysis

Samples were analyzed on a BD Accuri™ C6 Flow Cytometer (BD Biosciences, San Jose). A medium flow (34.5 µl/min) was used in 2 minutes collections. For small cells like bacteria, the medium flow was recommended due to higher accuracy. The blue laser (excitation 488 nm) was used. Cells stained with SYBR®Green II passes the blue laser, and the light is

collected by a detector (FL1) that reads blue laser excited emissions. FL1 detects emission within 533±15 nm. Emission maximum of the SYBR®Green II is 520 nm, well within the range of the detector. The intensity of fluorescent detected with FL1, represents the RNA content of individual cells. All samples were analyzed with the same settings.

Prior to analysis of the samples, the SYBR®Green II –RNA complex was tested for its fluorescent stability, to determine how fast samples must be analyzed after staining. One random pure culture was used. The flow cytometer had the capability to run 24 samples each run. The sample was first diluted 1:20 in filtrated seawater, enough to disperse 990 µl into 24 different plastic tubes. Then, 10 µl/ml SYBR®Green II (1:100) was added to the sample and mixed well with Paramix 3 (Julabo, Seelbach). The stained sample was then distributed into 24 plastic tubes (replicates) and incubated in the dark for 15 minutes.

During analysis, every other sample tube was covered with aluminum foil, whereas the other was exposed to light. This was to evaluate the effect light has on the stability of the dye. The total analysis time for 24 samples was 83 minutes. After evaluation of the stability, all samples analyzed in this project were analyzed within the stability period.

2.5.1.4 Data analysis of obtained flow cytometry results

The results from the pure samples and the microbial communities analyzed with flow cytometry, were processed with the BD Accuri™ C6 Software v.1.0.264.21 (BD Biosciences, San Jose). In the analysis of the diagrams, aggregated (multiple) bacteria is not representative for RNA content of single cells. Signal over 10^5 in forward scatter (FSC) in all the plots was thus excluded. The cut off and lower limit on the FL1 (RNA content) intensity were set to be 10^4. Fluorescent intensity below 10^4 was considered noise and viruses that are smaller than bacteria, and has not been included in the analysis.

To set the fluorescent thresholds to determine the fraction of r- or K-strategists within a microbial community, the BD Accuri™C6 data file resulting from the flow cytometry analysis of surface and 90 meter seawater samples was imported into FSC Express 5 (https://www.denovosoftware.com/). The fluorescent signal (FL1) was divided into 12 logarithmic subdivisions and evaluated to set the suitable fluorescent thresholds.

2.5.2 DNA sampling, extraction and concentration determination

2.5.2.1 DNA sampling

Sampling from the pure cultures and ATCC reference strains were done by centrifugation of the cultures at 6000 rpm for 3 minutes in Himac CT15E (Hitachi, Ltd., Tokyo). The supernatant were thrown away and the pellet was frozen at -20°C for DNA isolation. For the communities where a shift from K-to r-selection was established, 50 ml from the cultivation flasks was sucked into 50 ml sterile disposable pipettes (BD Biosciences, San Jose). Syringe tip filter (Specrum®Laboratories, Inc., Rancho Dominguez) with 0.2 µm pores was attached to the tip, and the water was filtrated through. Filters were stored at -20°C until DNA isolation.

2.5.2.2 DNA extraction with Power Soil® DNA Isolation Kit

To isolate genomic DNA from the samples, Power Soil® DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad) was used. The kit relies on a bead beating tube for a rapid homogenization of the sample. By chemical and mechanical methods, the cells were lysed and the total genomic DNA was captured on a silica membrane in a spin column. Several washing steps were included and a final elution step released the isolated DNA from the spin column.

The procedure of the isolation was performed as in the manual of Power Soil® DNA Isolation Kit (Appendix D). For pure cultures, the bacterial pellets were used, whereas for mixed communities the filter samples were used. The filters were removed from its holder and transferred directly into the first tube of the kit. The isolated DNA was frozen at -20°C until determination of DNA concentration.

2.5.2.3 Determination of DNA concentration by Qubit® fluorometer 2.0

Concentration of all DNA extracted samples was determined using Qubit® 2.0 Fluorometer (Life Technologies, Thermo Fisher Scientific Inc., Waltham). Qubit ™ dsDNA HS assay kit (Life Technologies, Thermo Fisher Scientific Inc., Waltham) was used in combination with the Qubit® 2.0 Fluorometer to determine DNA concentration of DNA extracts. The kit contains two standards, dilution buffer and a dye that becomes fluorescent when it binds to

double-stranded DNA (dsDNA). The two standards with known concentrations were used to construct a standard curve, from which the DNA concentration of all the samples was calculated from. The measurements were conducted according to the manual of Qubit ™ dsDNA HS Assay Kit (see Appendix E). All samples were run on the same day, with the same working solution and standards. This was done to minimize the source of error.

2.5.3 Real-time quantitative PCR

PCR primers targeting conserved regions of the bacterial 16S rRNA gene had previously been designed in the research group. Primer sequence and melting temperatures (Tm) are given in Table 2.1.

Table 2.1: Primers used in the rtPCR reaction with respective sequence, supplier and melting temperature (Tm).

Primer	Supplier	Tm	Sequence
533R	Sigma Aldrich	67.5°C	5`- TTACCGCGGCTGCTGG
339F	Sigma Aldrich	66.6°C	5`-TCCTACGGGAGGCAGCAG

The Power SYBR® Green PCR master mix (Applied Biosystems, Thermo Fisher Scientific Inc., Waltham) contains all the components needed in the reaction, excluding the primers and the template. This includes AmpliTaq Gold® DNA Polymerase and a blend of dTTP/dUTP. It also includes a ROX dye as a passive reference. This normalizes the non-PCR fluorescence variability in the wells.

The reaction mix was prepared using 12.5 µl Power SYBR® Green PCR master mix, adding forward and reverse primers to a total concentration 0.5 µM of each. In addition, sterile water was added to a final volume of 20 µl for each sample. The reaction master mix was used for all the samples in each run. For each reaction, reaction master mix was added to MicroAmp ™ Optical 8-tube strip (Applied Biosystems, Thermo Fisher Scientific Inc., Waltham). To each respective well, template was added, creating a total reaction volume of 25 µl. Triplicate reactions of each sample were performed.

The rtPCR reactions were run in a 7500 Real-time PCR instrument (Applied Biosystems, Thermo Fisher Scientific Inc., Waltham). The following cycling conditions were used:

Holding stage	50°C	2 min.	
Denaturation	95°C	10 min.	
Denaturation	95°C	15 sec.	40 cycles
Annealing/Extension	60°C	1.5 min.	

The melting curve analysis was performed on the PCR products when the amplification was finished, by using the following temperatures:

15°C	15 sec.
60°C	1 min.
95°C	30 sec.
60°C	15 sec.

Before analysing the samples, amplification efficiency of 16S rRNA primer pair, specificity of the amplification and optimal template amount were determined by construction of standard curves from dilution series. 1:5 dilution series (0.0008 to 0.5 ng) of DNA from two different pure strains were made. The obtained cycle threshold (Ct) values from the analyzed dilution series were plotted agains quantity of template DNA (log scale). A linear regression was performed on the standard curve. Amplification efficiency was calculated by using the formula: $E= (10^{(-1/slope)})$. An amplification efficiency (E) of 2 is equivalent to 100% efficiency.

Same amount of template DNA was used in each reaction for all the samples in this project, to normalize the PCR reactions. This was due to the lack of a suitable one-copy reference gene for normalization.

2.5.3.1 Estimation of 16S rRNA gene copy number per genome

To estimate the 16S rRNA gene copy number per genome from the obtained real-time PCR (rtPCR) data, the difference in amplification efficiency between each rtPCR reaction must be corrected for. The data files from the rtPCR analysis of the pure cultures, was imported from the instrument into LinRegPCR computer program v.2014.8 (http://linregpcr.hfrc.nl/). A linear regression was performed, to correct for differences in amplification efficiency between each reaction in the 16S rRNA gene copy number determination. Mean Ct and mean amplification efficiency were based upon the LinRegPCR analysis of the three replicates, with deviants excluded. A normalized copy number was then calculated from the mean amplification efficiency (E) and mean Ct-value obtained from the LinRegPCR analysis by using the following equation:

$$\text{Copy number per genome} = E^{-Ct}$$

The same amount of template DNA was used in all reactions. The reference strain *B.licheniformis* was known to exhibit 7 copies of the 16S rRNA gene and was used to perform a relative quantification. The estimated 16S rRNA gene copy number determination of the pure strains, was thus normalized to total DNA template input and relative to the reference strain *B.licheniformis*. For the communities (surface and 90 meter water), the estimated 16S rRNA gene copy number from the triplicate reactions, was given relative to the lowest estimated 16S rRNA copy number. This was defined as one copy number of 16S rRNA gene.

2.5.4 PCR amplification and DNA sequencing of 16S rDNA in pure r- and K-strategists

To classify and assign taxonomy to the pure isolated strains, a region of the 16S rRNA gene was sequenced. Sanger sequencing was performed at GATC Biotech AG (Constance, Germany). Amplification and purification of the PCR products were required prior to shipment.

A traditional end-point PCR was run performed to amplify an approximately 500 bp (base-pair) region in the 5`end using the primers 8F-GC-M13R and 518R (see Table 2.2 for sequences). The reactions were run with Taq-polymerase with accompanying reaction buffer

(VWR International, Radnor), 2.0 mM $MgCl_2$, 0.3 µM of each primer, 200 µM of each dNTP and 1 ng DNA template.

Table 2.2: Sequences and supplier of the primers used for the PCR amplification and sequencing 5`-region of the 16S rRNA gene.

Primer	Supplier	Sequence
8F-CG-M13R	Eurofins MWG Operon	5`CAGGAAACAGCTATGACCGCCCGCCG CGCGCGGCGGGCGGGGCGGGGGCACGG GGGGACTCCTAGGGAGGCAGCAG
518R	Eurofins MWG Operon	5`- ATTACCGCGGCTGCT
M13R	Eurofins MWG Operon	5`CAGGAAACAGCTATGACC

The PCR reaction was carried out on Arktik Thermal Cycler (Thermo Fisher Scientific Inc., Waltham) on following cycling conditions:

Denaturation	95°C	3 min.	
Denaturation	95°C	30 sec.	35 cycles
Annealing	50°C	30 sec.	
Extension	72°C	60 sec.	
Final extension	72°C	10 min.	

Due to a low quantity of PCR products, some of the samples were run with 40 cycles (R3 and K23).

To check the quality and quantity, the PCR products were subjected to agarose gel electrophoresis. The 1% agarose gel was made by adding 4 g of agarose in 400 ml 1X Tris-acetate (TAE) buffer (Appendix F) and boiled in the microwave oven. The gel was cooled until 65°C and then added 20 µl GelRed (Biotium Inc., Hayward). In each well, 5 µl PCR product and 1 µl loading buffer (Thermo Fisher Scientific Inc., Waltham) was added. Gene Ruler 1kb Plus DNA ladder (Thermo Fisher Scientific Inc., Waltham) was included in the first and last well as a reference.

The large gel was run in 1x TAE buffer at 140 volts for 45 minutes, whereas the rerun of R3 and K23 was done in a small gel using 100 volts for 45 minutes. The gel was visualized under UV light and photographed in G:box (Syngene, Cambridge) by using the program GeneSnap (Syngene, Cambridge).

PCR products were purified using the QIAquick PCR purification kit (Qiagen, Valencia, USA) for removing PCR primers, nucleotides, polymerase and salt. The protocol is given in Appendix G. M13R (Table 2.2) was used as sequencing primer. The PCR products (100-400 ng) were mixed with the sequencing primer (2.5 µM in total concentration) and shipped to GATC Biotech AG. The resulting sequences were classified using the Classifier function of the Ribosomal Database Project (Wang *et al.*, 2007). When classifying the strains, a confidence threshold >80 % gives the lowest taxonomic level with a reliable classification.

The chromatogram for each PCR product was examined using Chromas Lite v.2.1.1 (http://technelysium.com.au/) to evaluate quality. To compare sequences that were classified as the same taxon, sequence alignment were constructed with the European Bioinformatics Institute`s computer program ClustalW2 (Larkin *et al.*, 2007).

2.5.5 Test of cpn60 primers for potential digital droplet analysis

Chaperonin-60 gene (*cpn60*) is a highly conserved single-copy gene which might be a candidate gene for normalization of the rtPCR 16S rDNA results. Universal bacterial PCR primers targeting this gene have previously been designed and the gene has been suggested as an alternative to the 16S rRNA gene in analysis of microbial diversity (Schellenberg *et al.*, 2011). The cpn60 primer cocktail (Schellenberg *et al.*, 2011), consisted of two forward and two reverse primers (Table 2.3), and was used to test the primer`s ability to amplify a specific fragment of *cpn60*. The primers were used in a traditional end-point PCR, but with standard reaction and cycling conditions for the digital droplet PCR (ddPCR).

Table 2.3: Cpn60 primers with respective sequence, supplier and melting temperature (Tm). I= Inosine ,Y= C+T, R= A+G.

Primer	Supplier	Tm	Sequence
H279F	Sigma Aldrich	37.5°C	5`- GAIIIIGCIGGIGAYGGIACIACIAC
H280R	Sigma Aldrich	55.8°C	5`- YKIYKITCICCRAAICCIGGIGCYTT
H1612F	Sigma Aldrich	52.1°C	5`-GAIIIGCIGGYGACGGYACSACSAC
H1613R	Sigma Aldrich	80.1°C	5`-CGRCGRTCRCCGAAGCCSGGIGCCTT

QX200™ ddPCR™EvaGreen® Supermix (Bio-Rad Laboratories, Inc., Hercules, USA) was used for the PCR reactions (12.5 µl). The supermix contains all the components needed in the reaction, except the primers and the template. The total concentration of primers in the PCR reaction was 0.5 µM of both the forward and the revers primer mix. As recommended by Schellenberg *et al.* (2011), the forward primer mix contained 1/3 H279F and 2/3 H1612F, and the reverse primer mix contained 1/3 H280R and 2/3 H1613R. DNA from two pure strains was used as the template in the PCR reactions. Water and template (0.05 ng) were added to a final volume of 25 µl.

The PCR reactions were carried out in an Arktik Thermal Cycler (Thermo Fisher Scientific Inc., Waltham). A temperature gradient was applied to identify the optimal annealing temperature for the cpn60 primers. In addition, both the cpn60 primers and the 16S rRNA primers were tested at the standard temperature cycling suggested by the ddPCR supplier (Bio-Rad Laboratories, Inc., Hercules). Following cycling conditions were used:

Enzyme activation	95°C	5 min.	
Denaturation	95°C	30 sec.	38 cycles
Annealing	Varying*	30 sec.	
Extention	60°C	30 sec.	
Final extention	60°C	5 min.	

*Annealing temperatures are given in the Results, section 3.5.

After amplification, the PCR products were analyzed by agarose gel electrophoresis as described under section 2.5.4.

3. Results

Results are structured according to the two methods used, flow cytometry and real-time PCR , including results from both the pure strains and the mixed microbial communities. Sequencing of the pure cultures and test of primers for the potential digital droplet PCR method is presented separately from the two methods.

3.1 Isolation of r- and K-strategist from seawater according to growth rate

Pure bacterial strains with a variable maximum specific growth rate that represented the r- and K-strategists were isolated from surface seawater. In total, 24 strains of bacteria were isolated from the seawater, according to the time it took to form a colony (section 2.3.2). Counting of colonies was implemented every 24 hours for 12 days. 1/T determines the maximum specific growth rate (μ_{max}) (Salvesen and Vadstein, 2000). T is the time (hours) it took to form a visible colony. T was not precisely determined, because the counting of colonies was performed in a 24-hour interval. The isolated strains with their corresponding ID, $\mu_{max}h^{-1}$ (1/T), the definition as K-or r-strategist, and the taxonomic classification is given in Table 3.1.

Table 3.1: The 24 pure strains isolated from agar plates are given with respective parameters and taxonomic classification. Time to form a visible colony is given in days.

Id	Days (T)	1/T ($\mu_{max}h^{-1}$)	Defined as	Taxonomy
R1	2	0.0208	r-strategist	*Pseudoalteromonas*
R2	2	0.0208	r-strategist	*Vibrio*
R3	2	0.0208	r-strategist	*Pseudoalteromonas*
R4	2	0.0208	r-strategist	*Pseudoalteromonas*
R5	2	0.0208	r-strategist	*Pseudoalteromonas*
R6	2	0.0208	r-strategist	*Pseudoalteromonas*
R7	2	0.0208	r-strategist	*Pseudoalteromonas*
R8	2	0.0208	r-strategist	*Vibrio*
R9	2	0.0208	r-strategist	*Vibrio*
R10	2	0.0208	r-strategist	*Vibrio*
R11	2	0.0208	r-strategist	-
R12	3	0.0139	r-strategist	*Pseudoalteromonas*
R13	3	0.0139	r-strategist	*Pseudoalteromonas*
R14	3	0.0139	r-strategist	*Pseudoalteromonas*
U15	4	0.0104	Undefined	*Pseudoalteromonas*
U16	4	0.0104	Undefined	*Pseudoalteromonas*
K17	5	0.0083	K-strategist	*Pseudomonas*
K18	5	0.0083	K-strategist	*Aquimarina*
K19	6	0.0069	K-strategist	*Thalassobacter*
K20	7	0.0060	K-strategist	*Thalassobacter*
K21	7	0.0060	K-strategist	*Aquimarina*
K22	8	0.0052	-	-
K23	11	0.0038	K-strategist	*Pacificibacter*
K24	12	0.0035	K-strategist	*Pacificibacter*

The pure strains with a visible colony after five days (120 hours) were considered to be slow-growing K-strategist (Table 3.1). The strains that used up to three days (72 hours) to form a visible colony were considered fast-growing r-strategists. The isolated strains used throughout the experiments consisted of 7 K-strategists and 14 r-strategists. Two strains (U15 and U16) were not defined, as they were considered to be somewhere in between r- and K-strategists. Strain K22 was not used further in the project, due to no registred growth when incubated with medium. Strain R11 was contaminated during PCR amplification prior to sequencing and thus has no taxonomic assignment.

3.2 Sequencing of the 16S rDNA for r- and K-strategist

The pure cultures representing r- and K-strategists (Table 3.1) were sequenced for taxonomic classification and to compare 16S rRNA gene copy number with previously reported findings. Nineteen of the strains were classified at the genus level. For the remaining strains (K19, K20 and K23), the classification at the genus level is suggested, but at a confidence threshold below 80% (Table 3.2).

Table 3.2: Taxonomic classification of the isolated pure strains (R1-K24).

Sample	Phylum	Class	Order	Family	Genus
R1	*Proteobacteria*	*Gammaproteobacteria*	*Alteromonadales*	*Pseudoalteromonadaceae*	*Pseudoalteromonas*
R2	*Proteobacteria*	*Gammaproteobacteria*	*Vibrionales*	*Vibrionaceae*	*Vibrio*
R3	*Proteobacteria*	*Gammaproteobacteria*	*Alteromonadales*	*Pseudoalteromonadaceae*	*Pseudoalteromonas*
R4	*Proteobacteria*	*Gammaproteobacteria*	*Alteromonadales*	*Pseudoalteromonadaceae*	*Pseudoalteromonas*
R5	*Proteobacteria*	*Gammaproteobacteria*	*Alteromonadales*	*Pseudoalteromonadaceae*	*Pseudoalteromonas*
R6	*Proteobacteria*	*Gammaproteobacteria*	*Alteromonadales*	*Pseudoalteromonadaceae*	*Pseudoalteromonas*
R7	*Proteobacteria*	*Gammaproteobacteria*	*Alteromonadales*	*Pseudoalteromonadaceae*	*Pseudoalteromonas*
R8	*Proteobacteria*	*Gammaproteobacteria*	*Vibrionales*	*Vibrionaceae*	*Vibrio*
R9	*Proteobacteria*	*Gammaproteobacteria*	*Vibrionales*	*Vibrionaceae*	*Vibrio*
R10	*Proteobacteria*	*Gammaproteobacteria*	*Vibrionales*	*Vibrionaceae*	*Vibrio*
R11	*Proteobacteria*	*Gammaproteobacteria*	*Alteromonades*		
K12	*Proteobacteria*	*Gammaproteobacteria*	*Alteromonades*	*Pseudoalteromonadaceae*	*Pseudoalteromonas*
K13	*Proteobacteria*	*Gammaproteobacteria*	*Alteromonades*	*Pseudoalteromonadaceae*	*Pseudoalteromonas*
K14	*Proteobacteria*	*Gammaproteobacteria*	*Alteromonades*	*Pseudoalteromonadaceae*	*Pseudoalteromonas*
K15	*Proteobacteria*	*Gammaproteobacteria*	*Alteromonades*	*Pseudoalteromonadaceae*	*Pseudoalteromonas*
K16	*Proteobacteria*	*Gammaproteobacteria*	*Alteromonades*	*Pseudoalteromonadaceae*	*Pseudoalteromonas*
K17	*Proteobacteria*	*Gammaproteobacteria*	*Alteromonades*	*Pseudoalteromonadaceae*	*Pseudomonas*
K18	*Bacteroidetes*	*Flavobacteriia*	*Flavobacteriales*	*Flavobacteriaceae*	*Aquimarina*
K19	*Proteobacteria*	*Alphaproteobacteria*	*Rhodobacterales*	*Rhodobacteraceae*	*Thalassobacter*
K20	*Proteobacteria*	*Alphaproteobacteria*	*Rhodobacterales*	*Rhodobacteraceae*	*Thalassobacter*
K21	*Bacteroidetes*	*Flavobacteriia*	*Flavobacteriales*	*Flavobacteriaceae*	*Aquimarina*
K23	*Proteobacteria*	*Alphaproteobacteria*	*Rhodobacterales*	*Rhodobacteraceae*	*Pacificibacter*
K24	*Proteobacteria*	*Alphaproteobacteria*	*Rhodobacterales*	*Rhodobacteraceae*	*Pacificibacter*

The sequences alignments are given in Appendix H. For 7 of a total of 11 strains that were classified as *Pseudoalteromonas* (R1, R3, R4, R6, R13, U15 and U16), the 16S rDNA sequence similarity was found to be 98-99%. Thus, they might represent the same strain. The other strains representing *Pseudoalteromonas* (R5, R7 and R12) had almost identical 16S rDNA sequences and probably represent another strain. The strain R14 differed in several nucleotide positions from the other *Pseudoalteromonas* 16S rDNA sequences, and probably represents a third strain of the *Pseudoalteromonas*.

Of the four strains classified as *Vibrio* (R2, R8, R9 and R10), 2 variants of the 16S rDNA sequence were identified, which were identical except for a variable stretch of approximately 25 nucleotides in the 5`-end of the gene (Figure 3.1).

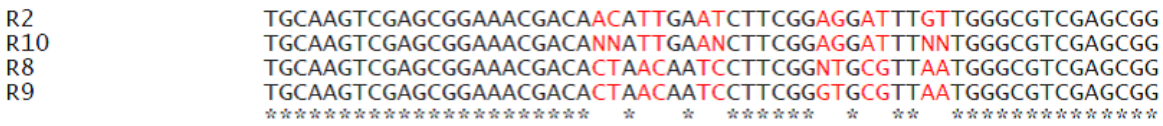

Figure 3.1: Extract of alignment of the four strains classified as *Vibrio* (R2, R8, R9 and R10)**.** The nucleotides marked with red represents the variable nucleotide positions.

The strains K19 and K20, classified *Thalassobacter*, was found to exhibit identical 16S rDNA sequences, and thus represents the same strain. The strains K23 and K24 were classified as *Pacificibacter*, and their 16S rDNA sequence differed in two nucleotide positions. The two strains (K18 and K21) classified as *Aquimarina*, were different in about 30 positions and in some positions, there were inserted bases. They may thus be two different strains.

Typical 16S rRNA gene copy number for the genera included in Table 3.2 was identified from The Ribosomal RNA Database (Table 3.3). In general, the fast-growing r-strategists classified as *Pseudoalteromons* and *Vibrio*, would be expected to contain 5 to 11 copies of the 16S rRNA gene in their genomes. The slow-growing K-strategist classified as *Aquimarina, Thalassobacter, Pseudomonas* and *Pacificibacter*, would be expected to contain between 1 and 7 copies of the gene in their genomes.

Table 3.3: Expected 16S rRNA gene copy number for the genera represented by the strains isolated in this project, determined by the Ribosomal RNA database (Stoddard and Smith, 2015). For the *Aquimarina (Flavobacteriaceae), Thalassobacter (Rhodobacteraceae)* and *Pacificibacter (Rhodobacteraceae)*, expected copy number is given according to family. This was due to no sequenced species on genus level in the database.

Family	Genus	Expected copy number of the 16S rRNA gene
Pseudoalteromonadaceae	*Pseudoalteromonas*	5-9
Vibrionaceae	*Vibrio*	6-11
Pseudoalteromonadaceae	*Pseudomonas*	4-7
Flavobacteriaceae		3-6
Rhodobacteraceae		1-5

3.3 Flow cytometry analysis for quantification of RNA content

3.3.1 Stability of SYBR®Green II when staining RNA complex in cells

To evaluate the stability of the SYBR®Green II - RNA complex and thus determine how fast samples should be analyzed after staining the RNA in cells, the isolated strain R13 was used. Replicates in 24 tubes were strained and analyzed by flow cytometry. All the replicates were incubated for 15 minutes prior to analysis in the dark, then analyzed with exposure or without exposure to light. Figure 3.2 shows the time until analysis (not including the 15 minutes of incubation) and the SYBR®Green II intensity (represented by FL1). The percentage decrease in fluorescent intensity in relation to the first of the replicates analyzed and the data is given in Appendix I.

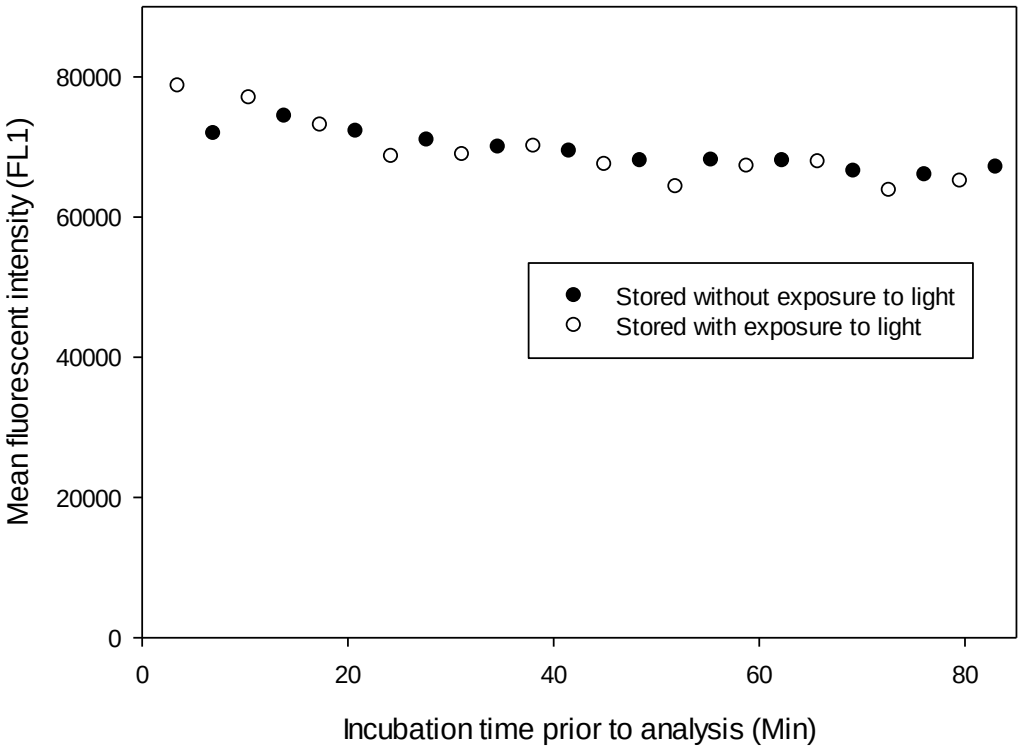

Figure 3.2: The mean fluorescent intensity of SYBR®Green II –RNA complex (collected with FL1) of the different samples as a function of incubation time (after the 15 minutes staining) and whether the samples were exposed to light or not.

There were relative similar results for measurements made in light and dark (Figure 3.2). In light, the decrease in intensity over time was below 10 % provided that the sample was analyzed within 17 minutes. When measurements were performed in darkness, the fluorescence appeared to be somewhat more stable over time. The decrease in fluorescence intensity was below 10% with an incubation time under 27 minutes in the dark. The second sample analyzed was an unexplainable outlier (Figure 3.2) and has not been used in the evaluation.

Based on the results above, all samples in this project were run within 27 minutes in the dark, to reduce error due to the decreased fluorescent intensity.

3.3.2 Optimization of flow cytometry analysis: Effect of temperature and incubation time on RNA content and cell division

The aim of the experiment was to define a suitable incubation time for different temperatures to create maximum cellular RNA content in a microbial community and avoid significant increases in cell numbers. Surface seawater from the Trondheimsfjord (Brattøra) was added nutrients and incubated at 12°C and 22°C, and sampling was performed in intervals within 24 hours (section 2.4.1). Cell number and RNA content (FL1) were determined by flow cytometry analysis of single cells (Table 3.4). The cellular RNA content should be measured before cell division, because the aim was to investigate the initial composition of the microbial community. Cell division causes a change in the composition, because fast-growing r-strategist divides quicker than the slow-growing K-strategist.

Table 3.4: Mean fluorescent signal (FL1) representing RNA content and cells/µl according to incubation time of the surface water at 12°C and 22°C incubation, analyzed by flow cytometry. Percentage increase in fluorescent signal was calculated relative to the sample before the medium addition (0 hour).

Sample	Time (Hour)	Cells/µl	Mean FL1	Percentage increase FL1
12°C	0	1 510	31 601	0.0
	1	1 500	31 389	-0.7
	2	1 580	31 731	0.4
	4	1 520	36 675	16.0
	8	1 420	44 749	41.6
	12	1 980	89 767	184.0
	24	36 410	159 160	403.5
22°C	0	1 870	32 538	0.0
	1	1 950	33 307	2.4
	2	1 990	34 571	6.3
	4	1 260	45 236	39.0
	8	3 000	127 760	292.7
	12	28 560	281 173	764.1
	24	88 710	133 997	311.8

The surface water incubated at 12°C had an increase in fluorescent signal (RNA content) per cell, but no increase in cell counts, at 8 hours of incubation (Table 3.4). That is an indication of a shift-up in the rRNA synthesis caused by nutrient addition, provoking the members to grow at a maximum specific growth rate. Between 8 and 12 hours, cell division of the fast-growing r-strategists caused an additional increase in cellular RNA content and a change in the microbial composition occurred (Figure 3.3). For the community incubated at 22°C, cell division of fast growing r-strategists occurred earlier than for sample incubated at 12°C, due to a higher incubation temperature (Figure 3.3). After 12 hours, the mean RNA content decreased again, probably due to nutrient limitation. Even though nutrient was limiting and growth rate decreased within the community, the cell counts were high due to dead/resting cells present.

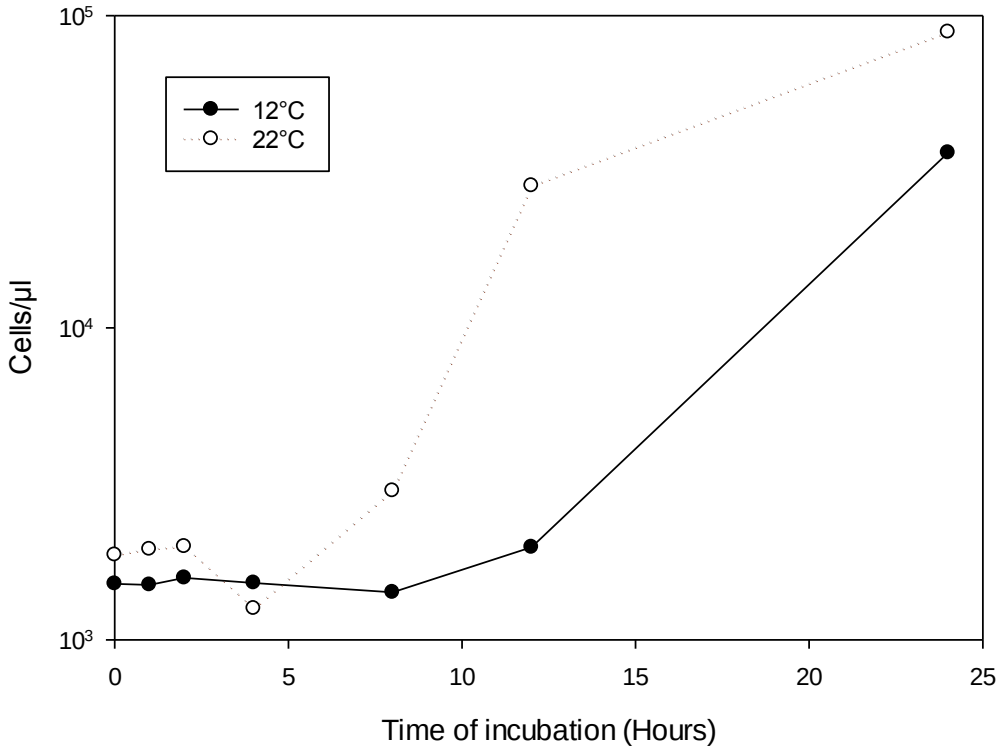

Figure 3.3: Cell counts (Cells/µl) in relation to incubation time (Hours) for the surface seawater at 12°C and 22°C incubation. The cells per µl axis are on a logarithmic scale.

The results of this experiment indicated that an incubation time of 4 hours at 22°C or 8 hours at 12°C was appropriate to achieve a maximum cellular RNA content in the communities, without significant cell division of fast-growing r-strategists.

3.3.3 Relationship between μ_{max}, RNA content and cell division in pure cultures of r- and K-strategists

The aim was to analyze pure cultures that represented r- and K-strategists (Table 3.1) with flow cytometry, to investigate if maximum RNA content per cell and maximum specific growth rate (μ_{max}) was correlated. Based on the results presented in section 3.3.2, pure cultures were incubated with medium at 15°C for 4 and 8 hours (see section 2.4.2).

The cell counts after 4 and 8 hours was determined, to evaluate the time for cell division of fast growing r-strategists compared to the slow growing K-strategists. The cell count per µl of the pure cultures after 4 and 8 hours of incubation were analyzed by flow cytometry, and the ratio of cell counts between 8 and 4 hours were calculated (Table 3.5).

Table 3.5: Cell counts determined by flow cytometry for all the pure cultures representing r- and K-strategists. 1/T is the maximum specific growth rate, where T is the time (hours) it took to form a colony. Number of cells/µl in all 23 samples is given after 4 and 8 hours of incubation. The ratio between cell counts at 8 and 4 hours are also given.

ID	1/T ($\mu_{max}h^{-1}$)	Cells / µL 4h	Cells / µL 8h	Ratio cells/µl (8h/4h)
R1	0.0208	335	1 450	4.33
R2	0.0208	330	1 460	4.42
R3	0.0208	350	1 065	3.04
R4	0.0208	185	835	4.51
R5	0.0208	185	1 210	6.54
R6	0.0208	295	435	1.48
R7	0.0208	310	1 470	4.74
R8	0.0208	210	4 320	20.57
R9	0.0208	345	6 180	17.91
R10	0.0208	575	10 820	18.82
R11	0.0208	215	1 500	6.98
R12	0.0139	190	835	4.40
R13	0.0139	620	1 780	2.87
R14	0.0139	235	970	4.13
U15	0.0104	10	10	1.00
U16	0.0104	555	2 725	4.91
K17	0.0083	18 250	18 550	1.02
K18	0.0083	10 330	14 920	1.44
K19	0.0069	11 580	8 280	0.72
K20	0.0060	7 110	7 530	1.06
K21	0.0060	15 890	21 490	1.35
K23	0.0038	880	910	1.03
K24	0.0035	540	600	1.11

As an indication of how fast the pure strains divide, the number of cell doublings in the r- and K-strategists between 4 and 8 hours of incubation with medium (Figure 3.4) was calculated by using the equation:

Doublings = Log_2 (Ratio 8h/4h)

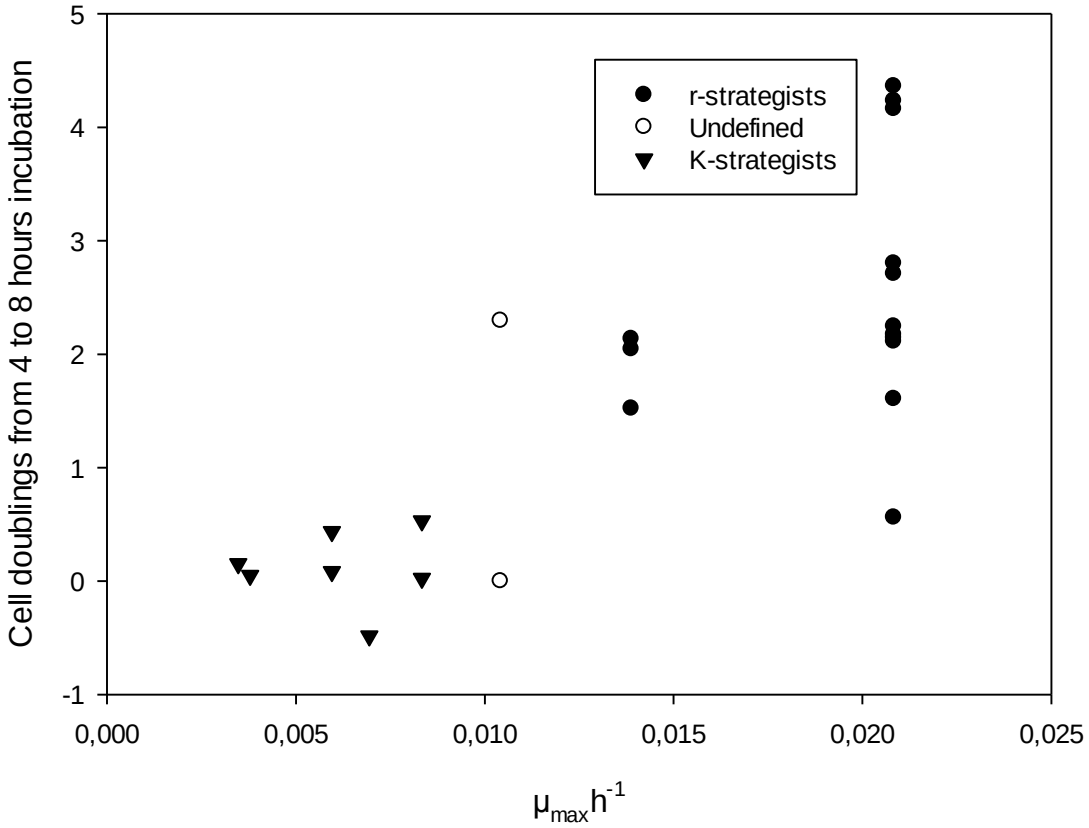

Figure 3.4: Number of cell doublings from 4 to 8 hours of incubation versus maximum specific growth rate ($\mu_{max}h^{-1}$) for each of the pure cultures representing r- and K-strategists.

The average cell doubling between 4 and 8 hours of the K-strategists was not significantly different from zero. (Figure 3.4). For the fast growing r-strategists, the cell doubling between 4 and 8 hours was positively correlated with maximum specific growth rate. Based on the results, the slow-growing K-strategists appeared to not divide within 8 hours incubation at 15°C, but the fast-growing r-strategists did.

In the flow cytometry analysis of the pure strain K19 at both 4 and 8 hours incubation, it appears like the strain consisted of two distinct microbial populations (Figure 3.5). In addition, the strain had a decrease in cell counts (Table 3.5). Cell division could thus have occurred, but due to the exclusion of aggregated cells (section 2.5.1.4) no doubling of cells was observed (Figure 3.5).

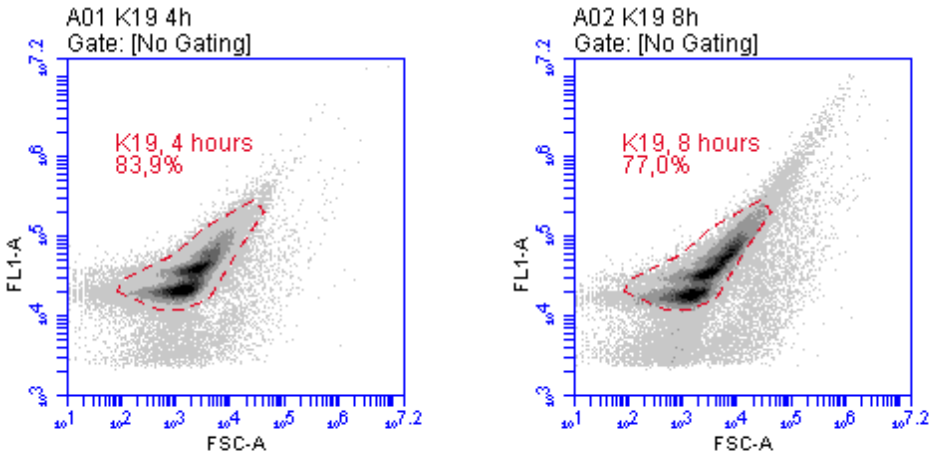

Figure 3.5: Flow cytometry diagrams with FL1 (representing RNA content) and FSC (representing cell size). Plots for sample K19 after 4 and 8 hours of incubation with the medium is shown. The cells within the red gating are included in the analysis.

Flow cytometry analysis conceived two distinct bacterial populations in several of the slow-growing K-strategist (K18-K21). For the other pure cultures the analysis indicated the existence of one bacterial population only. Different states of some of the cells, like lag phased or even dead cells, may not adapt within 8 hours of cultivation and thus not achieve maximum RNA content within the time period. If excluding the population assumed to consist of cells in lag phase/dead cells (lowest RNA content) of the samples with two populations, the mean RNA content per cell increases (Table 3.6). This indicates that some cells in the population had a low RNA content and may have been dead or in lag phase, whereas other cells were growing and have a higher RNA content.

Table 3.6: Samples containing two distinct bacterial populations (K18-K21) are given with mean fluorescent signal collected with FL1 (cellular RNA content), when including both populations and including only the population consisting of growing cells.

ID	Incubation time	Cellular RNA content, including two populations	Cellular RNA content, growing population
K18	4 hours	54 786	75 518
K18	8 hours	60 939	75 643
K19	4 hours	37 830	50 418
K19	8 hours	43 414	57 455
K20	4 hours	41 315	61 069
K20	8 hours	43 327	65 886
K21	4 hours	47 149	84 675
K21	8 hours	50 345	91 074

Due to the fact that dead/resting cells probably would not have an increase towards maximum RNA content within 8 hours of incubation, the consequence would be an underestimation of the maximum RNA content. Thus, the un-adapted cells were excluded, and the maximum RNA content per cell of the pure strains (K18-K21) was based only on the growing population.

Four of the pure cultures reached the maximum RNA content per cell after 4 hours. The remaining cultures had the highest RNA content per cell after 8 hours (Appendix J). The minimum RNA content per cell was determined for the pure cultures from stationary phase, where no growth was expected. The minimum RNA content per cell was relatively constant, except for two cultures, which had a high (K17) and low (K18) minimum RNA content compared to all the other pure cultures (Figure 3.6). The average fluorescent intensity, representing minimum cellular RNA content, was 40 705 ± 9187 (SD) (Figure 3.6), excluding the two outliers. A 95% CI (confidence interval) was thus between 39 740 and 44 670 in intensity. This indicates that the minimum RNA content of both r- and K-strategist is approximately the same.

The maximum RNA content of the r-strategists varied between $1.9 \cdot 10^5$ and $3.2 \cdot 10^5$, and for the K-strategists, between $2.4 \cdot 10^4$ and $1.6 \cdot 10^5$ (Figure 3.6).

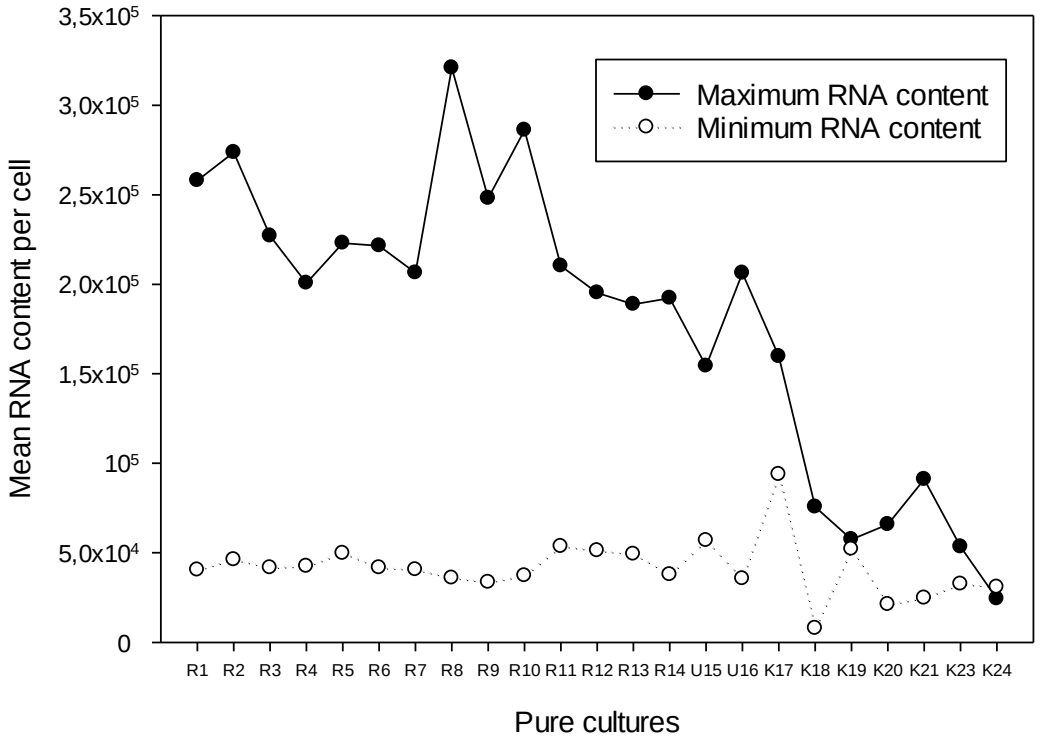

Figure 3.6: The relationship between the maximum cellular RNA content and the minimum RNA content for the 23 different pure cultures are shown.

The ratio between maximum and minimum cellular RNA content is shown in Figure 3.7 as a function of a proxy of maximum specific growth rate ($\mu_{max}h^{-1}$). A linear regression was implemented, excluding the deviating pure strain K18 (see Figure 3.7). Data of the ratios are given in Appendix J.

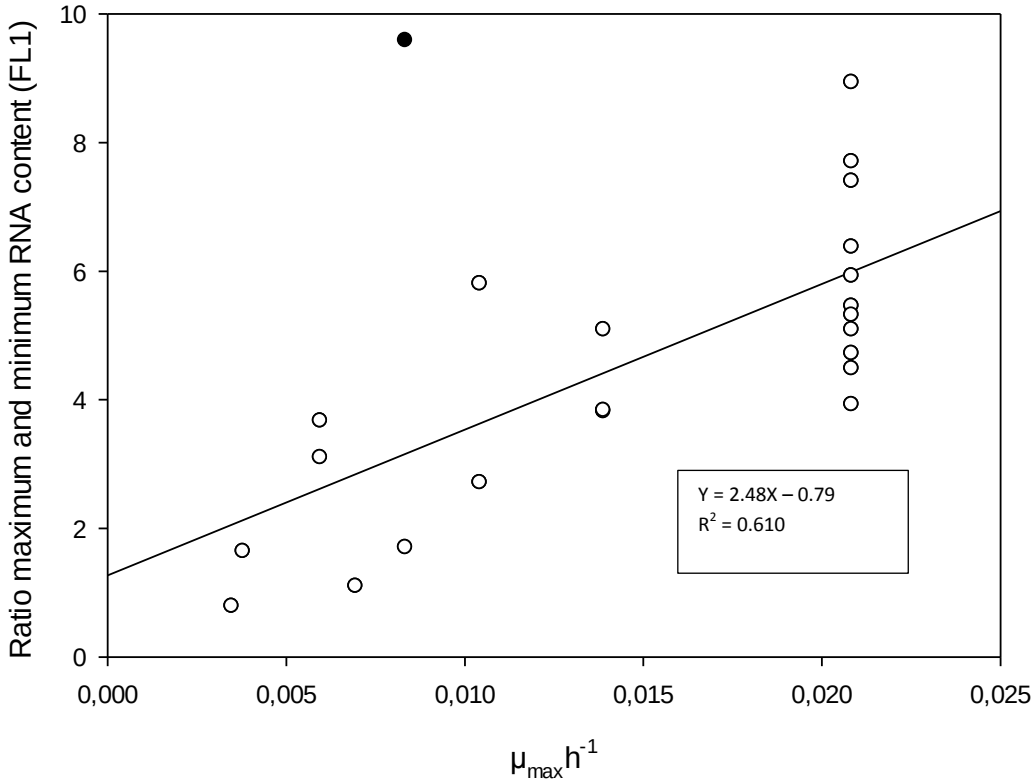

Figure 3.7: The ratios of the cellular maximum and minimum RNA content (collected with FL1) for pure strains representing r- and K-strategist according to maximum specific growth rate ($\mu_{max}h^{-1}$). The black point is the deviating culture K18 that was excluded from the linear regression

The results indicate that the higher maximum specific growth rate a bacteria possess, the larger increase in RNA content occurs when bacteria in a resting phase is triggered to reach μ_{max} (Figure 3.6, Figure 3.7).

3.3.4 RNA content and cell division in r- and K-selected communities

To investigate the RNA content of single cells in mixed r- and K-selected microbial communities, surface seawater and seawater collected at 90 meters from the Trondheimsfjord were used. To create a shift from K-to r-selection-to K-selection, nutrients were added at day 0 (initial shift). The hypothesis was that r-selection would be induced due to an excess of nutrients, and K-selection would be reintroduced when the added nutrients were consumed. On each sampling day, samples were taken out and incubated with nutrients (pulsing) to trigger a shift-up in the metabolism, inducing the cells to grow at maximum specific growth

rate and thus induce maximum RNA content per cell. Incubation of the samples at 15°C for 6 hours was used, based on the results obtained for the pure cultures (see section 2.4.3).

The surface seawater reached a higher cell count faster than the 90 meter seawater (Figure 3.8), when nutrition was added on day 0 (initial shift). This indicates a higher presence of fast-growing r-strategists in the surface seawater, compared to the 90 meter seawater, and is in accordance with expectations. Cell densities also decreased faster in the surface seawater. There was not large difference in cell counts between unpulsed (0 hours) and pulsed (6 hours) (Appendix K) in any of the communities, indicating that the incubation time and temperature were close to optimal. An exception was day 1 in both communities, as cell division was induced due to the excessive nutrients added on day 0 (initial shift).

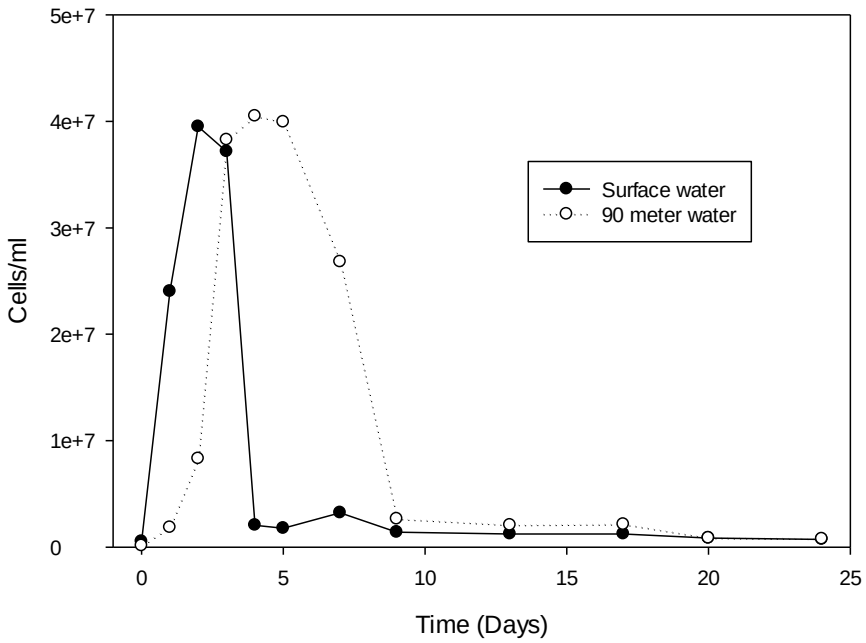

Figure 3.8: Cells per ml of the unpulsed samples on day 0-24, for both the surface and the 90 meter seawater samples.

After pulsing with nutrients, there was an increase in the maximum cellular RNA content in all days, except for day 1 (Figure 3.9). The lack of increase in RNA content on day 1 was probably due to excess of nutrients from the one time addition of medium on day 0 (initial shift).

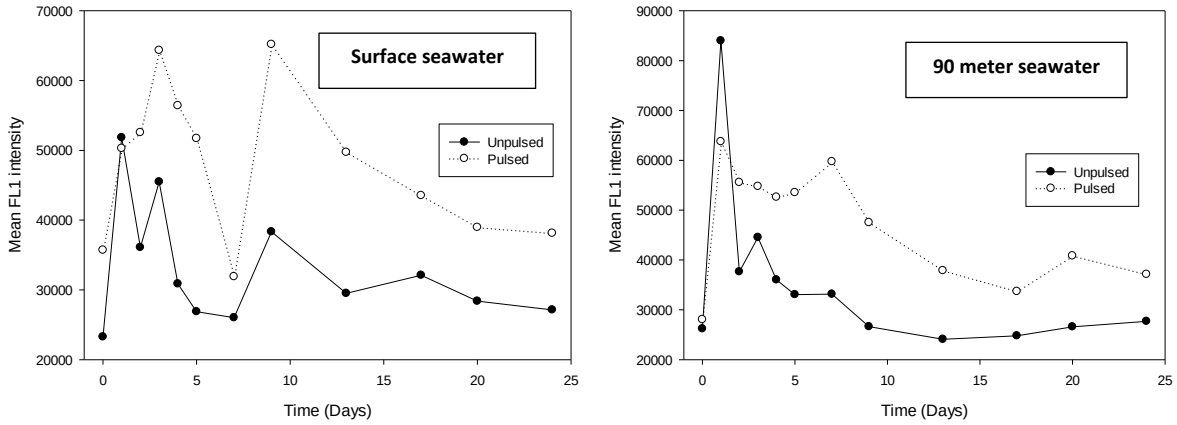

Figure 3.9: RNA content (FL1 intensity) for the surface and 90 meter seawater samples. The mean RNA content per cell in unpulsed (0 hours) and pulsed sample (6 hours) are illustrated for the incubation time between 0 and 24 days.

The increase in mean cellular RNA content after pulsing was low on day 0, probably due to K-selection in the two seawater samples. On day 1, when r-selected communities were expected, the mean RNA content per cell was higher in both communities. A gradual decrease in mean RNA content per cell was seen in both communities a few days after the maximum, indicating a succession due to K-selection. For the surface seawater, some outliers were observed (day 7 and day 9). Overall, these results from the surface and the 90 meter seawater samples indicated that pulsing with nutrients create a shift-up in the metabolism of the members in a community, and that the cellular RNA content increased toward a maximum after the pulse with nutrient and 6 hours incubation.

3.3.4.1 Distribution of cellular RNA content after shift-up and estimation of fraction of r- and K-strategists in microbial communities

The average fluorescent intensity cannot be used as an indicator of either r- or K-selected species, as such an indicator should include only cells with high or low RNA content, respectively. However, calculation of percentage r- or K-strategists was the aim of this study. A strategy could be to set a fluorescent threshold to determine the portion r- or K-strategists in the community as the percentage of cells above or below these two thresholds, respectively. Two criteria were used for determining a suitable threshold for calculation of percentage r-

and K-strategists within a community, based on the maximum RNA content of single cells after pulsing with nutrients and subsequent incubation:

- Assumption: The two seawater samples (surface and 90 meter) were K-selected on day 0. r-selection occurred after the addition of nutrients on day 0 (initial shift), and changed to K-selection when the added nutrients was consumed, indicated by a maximum in cell numbers.
- Dynamic variability: The percentage r- and K-strategist would ideally range between 0 and 100%. However, the threshold should be set in a way that maximizes the variability in percentage r- and K-strategists in the two seawater samples over the 24 days of incubation.

No objective criteria exist for setting the thresholds for r- and K-strategists, but fluorescence thresholds that meet the two criteria above serve as a pragmatic method. To identify fluorescent intensities that served as threshold for calculation of percentage r- and K-strategist, a two-step process was performed.

First, the frequency distribution of the fluorescent signal (FL1) from the flow cytometry analysis was made, was split into 12 logarithmic subdivisions by gridlines according to the intensity of FL1 signal for both unpulsed and pulsed samples (Figure 3.10). This was to calculate the fraction of cells within a given fluorescent intensity range (RNA content). To get an indication of the shift in RNA content for each of the subdivisions after the nutrient pulse, examples of the percentage distribution of fluorescent intensity are shown for three sampling days (Figure 3.11, Figure 3.12 and Figure 3.13). When K-selection was expected (day 0 and day 24), the shift-up in fluorescence was observed in subdivision 4 and 3, respectively (Figure 3.11 and Figure 3.13). Pulsing with nutrients generates a shift in the RNA content of the assumed r-selected community at a higher fluorescent intensity than for the K-selected communities (Figure 3.12). The shift between unpulsed and pulsed sample was observed in subdivision number 5. This indicates the presence of a higher fraction of fast growing r-strategist, with a higher RNA content per cell, compared to the K-selected communities at day 0 and day 24.

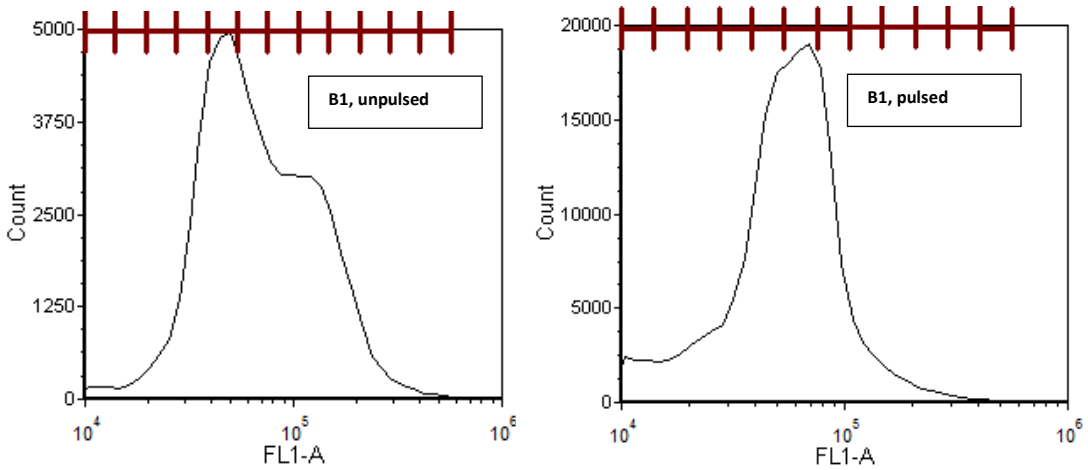

Figure 3.10: Distribution of fluorescent signal (RNA content) for cells determined by flow cytometry, for the 90 meter seawater on day 1 (B1), before and after a pulse with nutrients. The gridlines that determined the range of the 12 different subdivisions are indicated by red at the top of the plot.

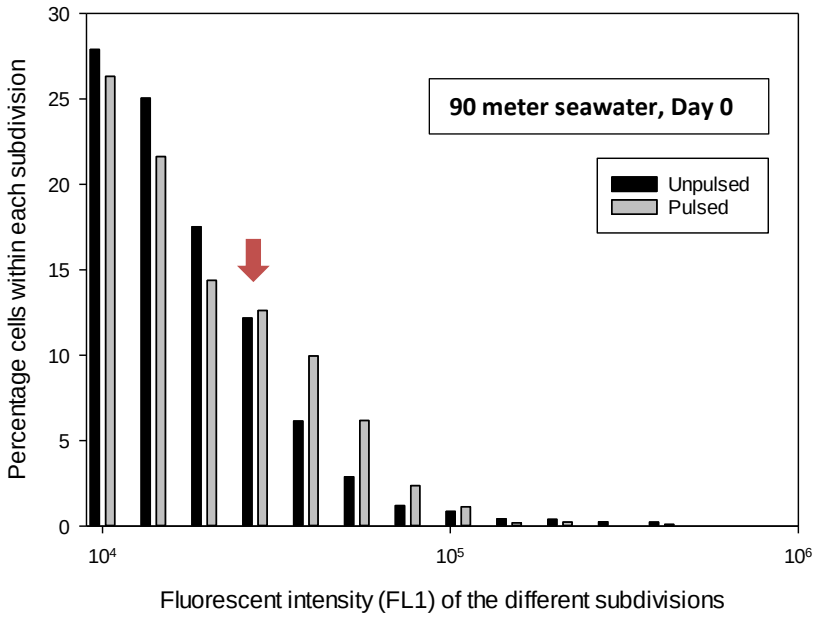

Figure 3.11: Frequency distribution of cellular fluorescent signal (RNA content) for the 90 meter seawater sample from day 0 (K-selection), before and after nutrient pulse. The red arrow indicates where the shift from unpulsed to pulsed sample happened.

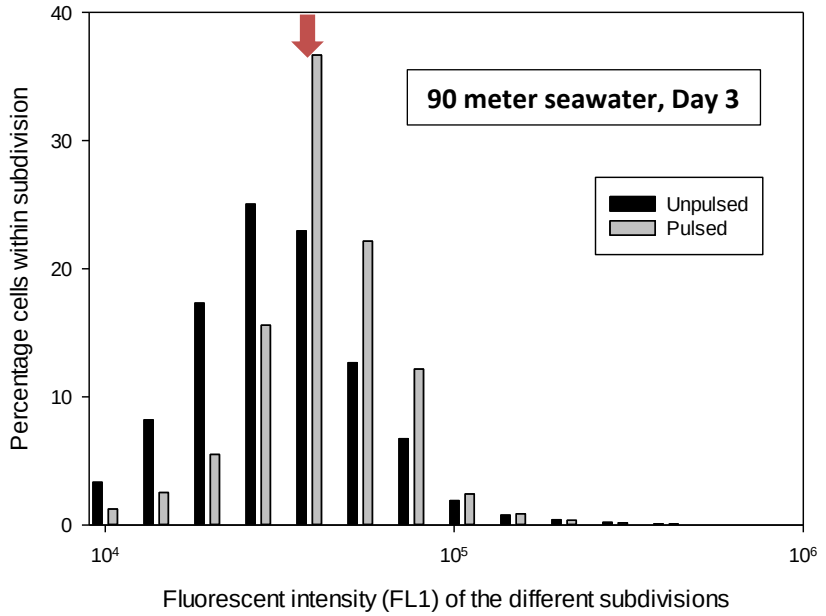

Figure 3.12: Frequency distribution of cellular fluorescent signal (RNA content) for the 90 meter seawater sample from day 3 (r-selection), before and after nutrient pulse. The red arrow indicates where the shift from unpulsed to pulsed sample happened.

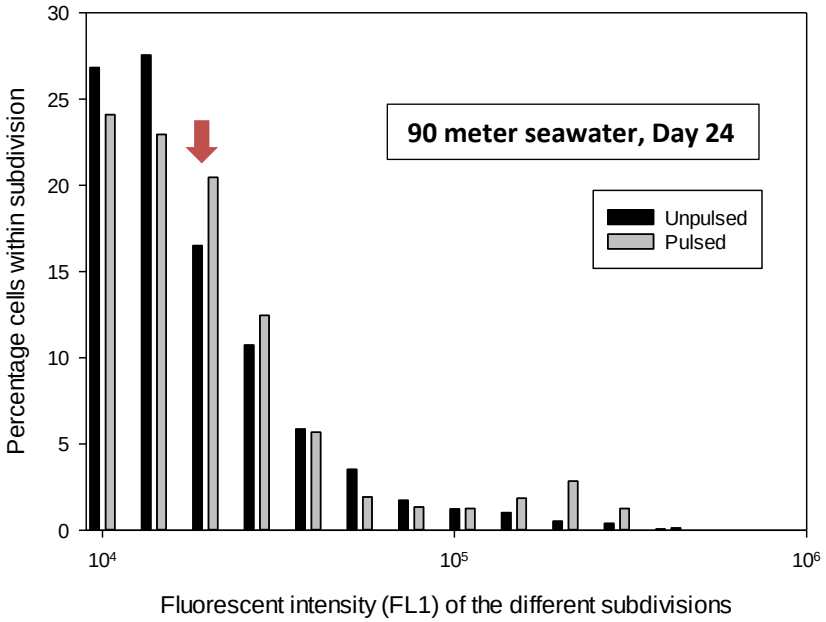

Figure 3.13: Frequency distribution of cellular fluorescent signal (RNA content) for the 90 meter seawater sample from day 24 (K-selection), before and after nutrient pulse. The red arrow indicates where the shift from unpulsed to pulsed sample happened.

Second, to decide thresholds in fluorescent intensity (maximum RNA content) after pulsing for calculating percentage r- and K-strategist, the assumption and dynamic variability criteria were used in the following way: Starting from the subdivision with the highest and the lowest fluorescent intensity for r- and K-strategist, respectively, the percentage r- and K-strategist were calculated. Then the neighbor subdivision was included and new percentages were calculated. This was continued until 8 subdivisions were included from the highest fluorescent intensity (percentage r-strategists) and 5 subdivisions included from the lowest fluorescent intensity (percentage K-strategists). Based on the number of subdivisions included, those percentages of r- and K-strategists that varies in accordance with the Assumption criteria, and has the highest difference between maximum and minimum percentage of r- and K-strategist during the experiment (Dynamic variability criteria), serves as suitable thresholds for calculation of r- and K-strategists. In reality, the threshold is then defined by the last subdivision included in the calculation. All fluorescent subdivisions with percentage cells, for both surface and 90 meter water are given in Appendix L, and percentage cells within included subdivisions in Appendix M.

With an assumption of K-selection in day 0 and 24, and r-selection around day 1-3, the percentage r-strategists should be considerably higher at day 1-3, compared to day 0 and 24. In both the surface and 90 meter seawater, the largest difference in percentage r-strategist in the r-selected communities (day 1-3) compared to K-selected communities (day 0 and 24), was observed when 7 and 8 subdivisions were included (Figure 3.14). The difference between minimum (day 0 and 24, K-selection) and maximum (day 1-3, r-selection) percentage r-strategists were over 36 %. For a lower number of subdivisions included, the difference was lower (6.7 to 22.0%), indicating a lower dynamic variability, compared to when 7 or 8 subdivisions were included. There was an unexpected decrease in percentage r-strategist in the surface seawater at day 4, 5 and 7, and an unexpected increase in day 9. The results from these days were thus not included in the evaluation.

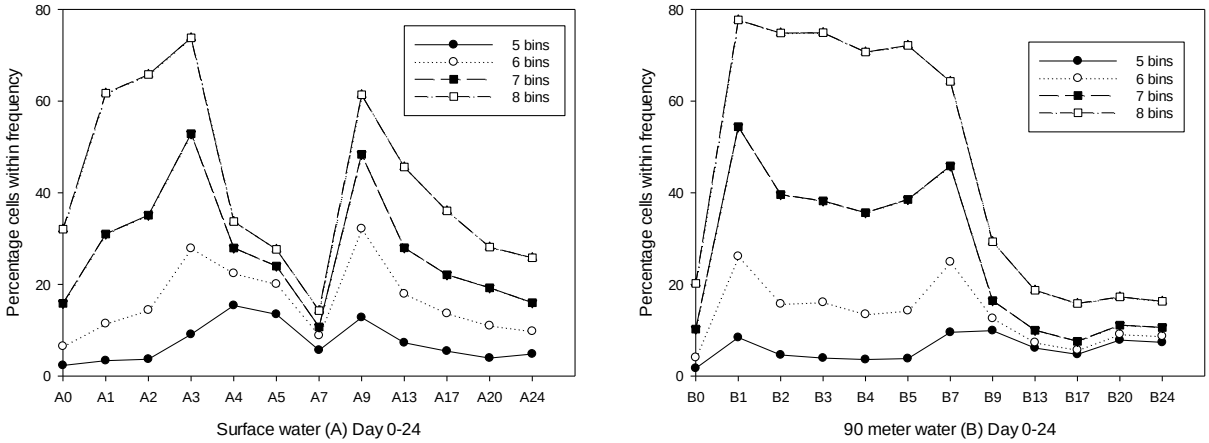

Figure 3.14: Percentage r-strategists in surface (A) and 90 meter seawater (B) communities, with number of fluorescent subdivisions (bins) added together from the highest fluorescent intensity and downwards. The four highest subdivisions are not shown.

For calculation of the percentage K-strategist, the assumption was a high fraction of K-strategist at day 0 and 24, due to K-selection, and lower percentage K-strategists on day 1-3 (r-selection). For the surface seawater samples, the largest difference between minimum (day 1-3) and maximum (day 0 and 24) percentage K-strategists was observed when 4 subdivisions were included, indicated by a difference of 43 % (Figure 3.15). For the 90 meter seawater, the difference was largest when 3 subdivisions were included, indicated by a difference of 60%. The difference in percentage K-strategists within the 24 days when a lower number of subdivisions were included was lower, which indicates a lower dynamic variability. The deviating days 4, 5, 7 and 9 in the surface seawater communities also affects the percentage K-strategists, and has not been included in the evaluation.

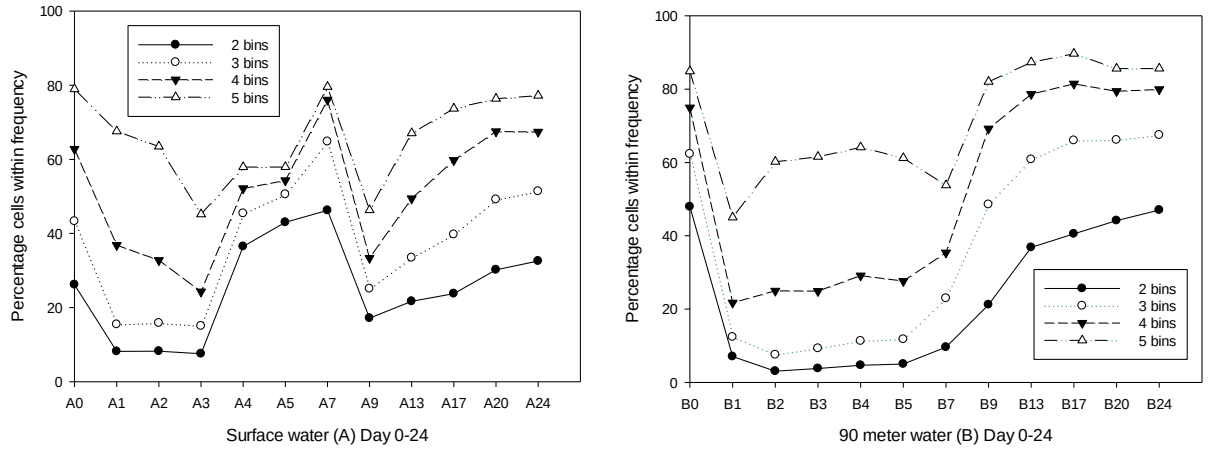

Figure 3.15: Percentage K-strategists in surface (A) and 90 meter seawater (B) communities, with number og fluorescent subdivisions (given as bins) added together from the lowest fluorescent intensity and upwards.

Based on the two criteria, the fluorescent thresholds for determining the fraction of r- and K-strategists can be proposed. There was similar dynamic variability with 7 and 8 fluorescent subdivisions included for the r-strategists and with 3 and 4 fluorescent subdivisions included for the K-strategists. The fluorescent intensity threshold of 27 440 (3 fluorescent subdivisions) is suggested for determination of K-strategists within a community. Cells with fluorescent intensities below 27 440 is thus considered to be K-strategists. For determination of percentage r-strategists within a community, a fluorescent intensity threshold of 53 782 (7 subdivisions) is suggested. Cells with fluorescent intensity above 53 782 is thus considered to be r-strategists. Moreover, with these two thresholds some cells are in between r- and K-strategist.

From the suggested thresholds, the fraction of K-strategist and r-strategist in the surface and 90 meter seawater over the time period of 24 days were calculated (Table 3.7). For the 90 meter seawater, dominance of K-selected species on day 0 was observed, with dominance of r-strategists on day 1 to around 7, and further a gradual succession to a dominance of K-strategists towards day 24. Similar results appeared for the surface seawater, with dominance of r-strategists on day 1-3 and gradually a succession towards dominance of K-strategists on day 13. Succession towards K-selection may have occurred earlier, but due to deviating results on day 4, 5, 7 and 9, it was a challenge to determine the exact time.

Table 3.7: Percentage K-strategists and r-strategists in the surface and the 90 meter seawater at day 0-24 after the nutrient pulse. The fraction of K-strategists were calculated by the percentage cells below a fluorescent intensity of 27 440. Percentage r-strategists were cells with a fluorescent intensity above 53 782.

	Percentage K-strategist		Percentage r-strategists	
Day	**Surface**	**90 meter**	**Surface**	**90 meter**
0	43.3	62.3	16.2	10.2
1	15.5	12.4	31.7	54.4
2	15.8	7.6	35.9	39.6
3	15.0	9.3	54.2	38.3
4	45.4	11.3	31.7	35.3
5	50.6	11.8	27.6	38.6
7	64.8	22.9	11.6	45.9
9	25.1	48.6	50.5	16.5
13	33.5	60.8	29.3	10.0
17	39.7	66.0	23.2	7.6
20	49.2	66.2	20.3	11.1
24	51.4	67.5	17.2	10.6

3.4 Real-time PCR analysis for quantification of 16S rRNA gene copy number

3.4.1 Test of 16S rRNA primers for real-time PCR

Real-time PCR (rtPCR) was used to estimate the 16S rRNA gene copy number per genome of pure bacterial strains representing r- and K-strategists and in mixed microbial communities. To test the amplification efficiency of the 16S rRNA primer pair, 1:5 dilution series (0.0008 to 0.5 ng) of DNA from two different pure strains were made (R6 and R8).

The resulting cycle threshold (Ct) values were plotted against quantity of template DNA (log scale). Linear regression was performed, and the resulting standard curve obtained for the R6 DNA is shown in Figure 3.16. The amplification efficiencies were calculated (see section 2.5.3) to be 102.83% and 101.95% respectively. Amplification efficiencies between 90% and 110% are considered acceptable (Thermo Fischer Scientific, 2014).

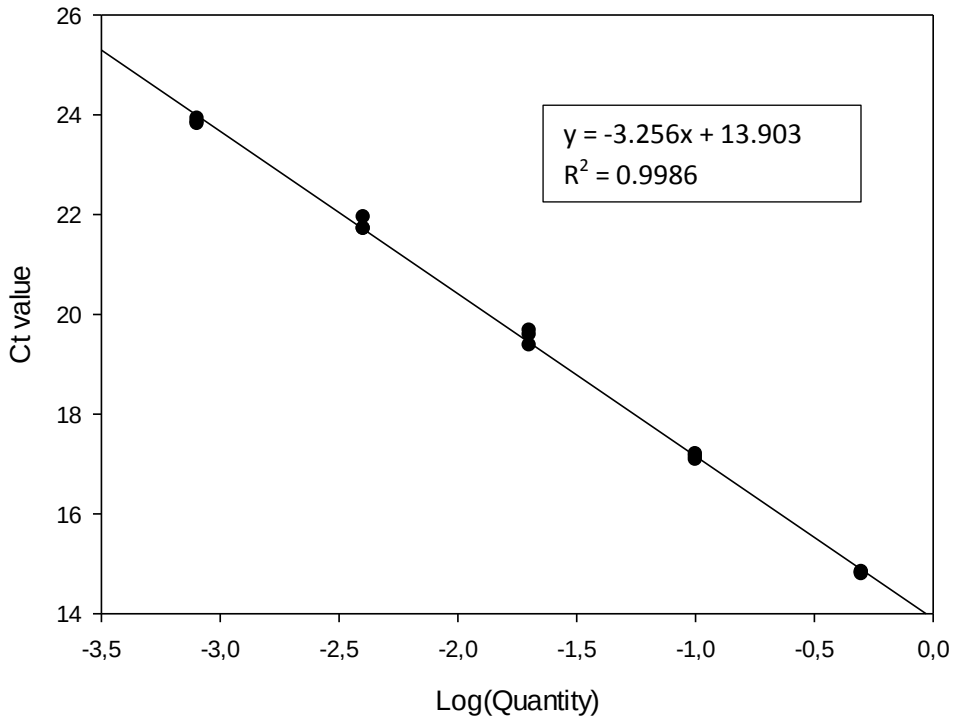

Figure 3.16: Standard curve for 1:5 dilution series of the pure strain R6 is shown. Triplicates of each concentration are shown. The slope (-3.256) was used to calculate amplification efficiency.

Melting curve analyses were performed to examine the PCR product obtained in the rtPCR reaction. At the lowest DNA concentration (0.008), the melting curve was broad and not uniform (Figure 3.17). This indicated that the template concentration was too low to obtain a high quality specific PCR product. The same trend was seen for both the pure strains analyzed. At higher DNA template concentrations, the melting curve showed only one peak and had a discrete form, indicating that the 16S rRNA primer pair used in the PCR reaction gives one specific product (Figure 3.17). The DNA template concentration should thus be between 0.004 to 0.5 ng. It was important to achieve only one product, as SYBR®Green II binds to all double-stranded DNA, also unspecific amplicons. In further rtPCR reactions, a total DNA concentration of 0.05 ng was used as template.

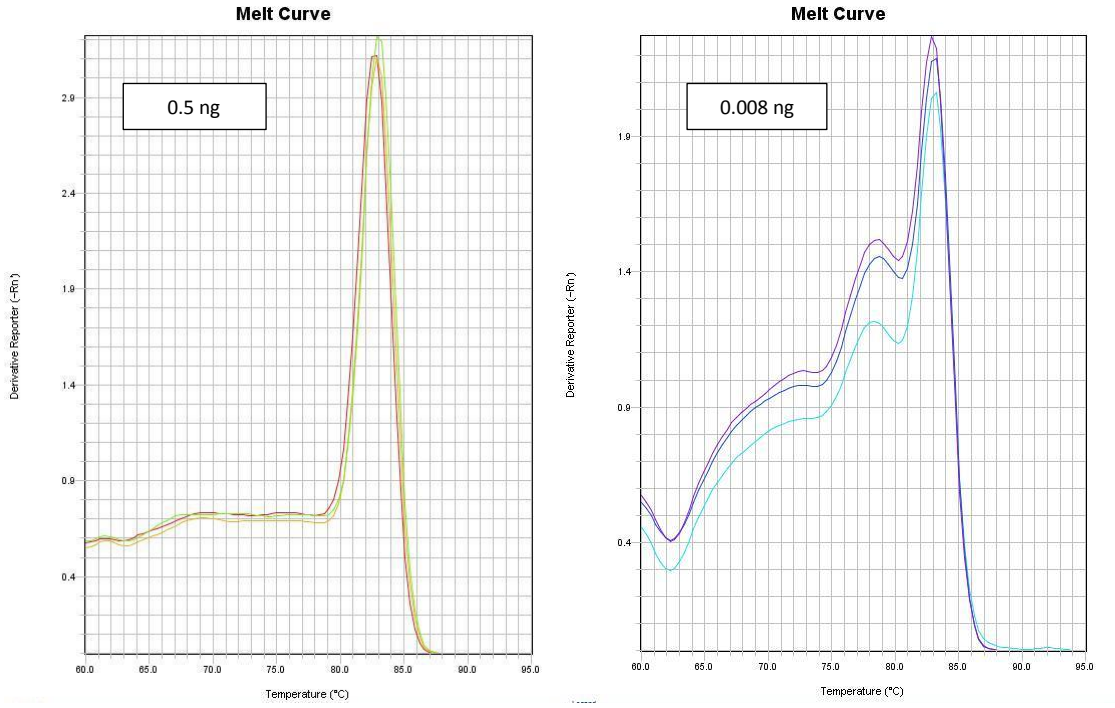

Figure 3.17: The melting curves for rtPCR amplicons obtained for strain R6. Curves for the highest (0.5 ng) and the lowest (0.0008 ng) DNA template concentration input in the rtPCR reactions are shown. Derivative reporter (-Rn) is the change in fluorescent divided by change in temperature and is plotted against temperature.

3.4.2 Relationship between μmax and 16S rRNA gene copy number of r- and K-strategists

The aim of the rtPCR analysis of the pure bacterial strains representing r- and K-strategist was to determine if 16S rRNA gene copy number and maximum specific growth rate (μ_{max}) are correlated. For each of the pure strains (Table 3.1), the relative 16S rRNA gene copy number was estimated (section 2.5.3.1) from the rtPCR results (Table 3.8).

Table 3.8: rtPCR data obtained for the pure strains with respective mean amplification efficiency E, mean Ct, and the estimated 16S rRNA gene copy numbers. Mean Ct and mean amplification efficiency were based on the three replicates, with deviants excluded.

Sample ID	Mean efficiency (E)	Mean Ct	E^{-Ct}	Estimated 16S rRNA gene copy number
R1	1.82	16.99	$3.96 \cdot 10^{-05}$	6.2
R2	1.86	16.70	$3.16 \cdot 10^{-05}$	5.0
R3	1.93	16.69	$1.70 \cdot 10^{-05}$	2.7
R4	1.86	16.98	$2.65 \cdot 10^{-05}$	4.1
R5	1.89	17.27	$1.69 \cdot 10^{-05}$	2.7
R6	1.84	17.01	$3.08 \cdot 10^{-05}$	4.8
R7	1.86	17.38	$2.01 \cdot 10^{-05}$	3.2
R8	1.89	16.83	$2.27 \cdot 10^{-05}$	3.6
R9	1.90	16.46	$2.61 \cdot 10^{-05}$	4.1
R10	1.92	16.58	$2.04 \cdot 10^{-05}$	3.2
R11	1.78	16.94	$6.01 \cdot 10^{-05}$	9.4
R12	1.83	17.11	$3.17 \cdot 10^{-05}$	5.0
R13	1.93	16.34	$2.15 \cdot 10^{-05}$	3.4
R14	1.90	16.80	$2.02 \cdot 10^{-05}$	3.2
U15	1.89	17.18	$1.85 \cdot 10^{-05}$	2.9
U16	1.87	16.98	$2.37 \cdot 10^{-05}$	3.7
K17	1.85	18.38	$1.19 \cdot 10^{-05}$	1.9
K18	1.87	18.46	$9.99 \cdot 10^{-05}$	1.6
K19	1.92	17.86	$8.71 \cdot 10^{-05}$	1.4
K20	1.85	17.94	$1.62 \cdot 10^{-05}$	2.5
K21	1.83	18.75	$1.16 \cdot 10^{-05}$	1.8
K23	1.88	17.66	$1.48 \cdot 10^{-05}$	2.3
K24	1.84	17.92	$1.87 \cdot 10^{-05}$	2.9
B.licheniformis	1.83	16.64	$4.47 \cdot 10^{-05}$	7.0
H.neptunium	1.71	20.31	$1.83 \cdot 10^{-05}$	2.9

The average amplification efficiency for the pure strain samples was 1.86±0.04 (SD). The estimated 16S rRNA gene copy number for the slow-growing K-strategist was ranging from 1.4 to 2.9, with an average of 2.0 (Figure 3.18). A higher 16S rRNA gene copy number was found for the fast-growing r-strategist, ranging from 2.7 to 9.4, with an average of 4.3.

The relationship between the estimated 16S rRNA copy number and the maximum specific growth rate ($\mu_{max}h^{-1}$) is visualized in Figure 3.18. The reference strains (*B.licheniformis* and *H.neptunium)* were not included, as the maximum specific growth rate was not known.

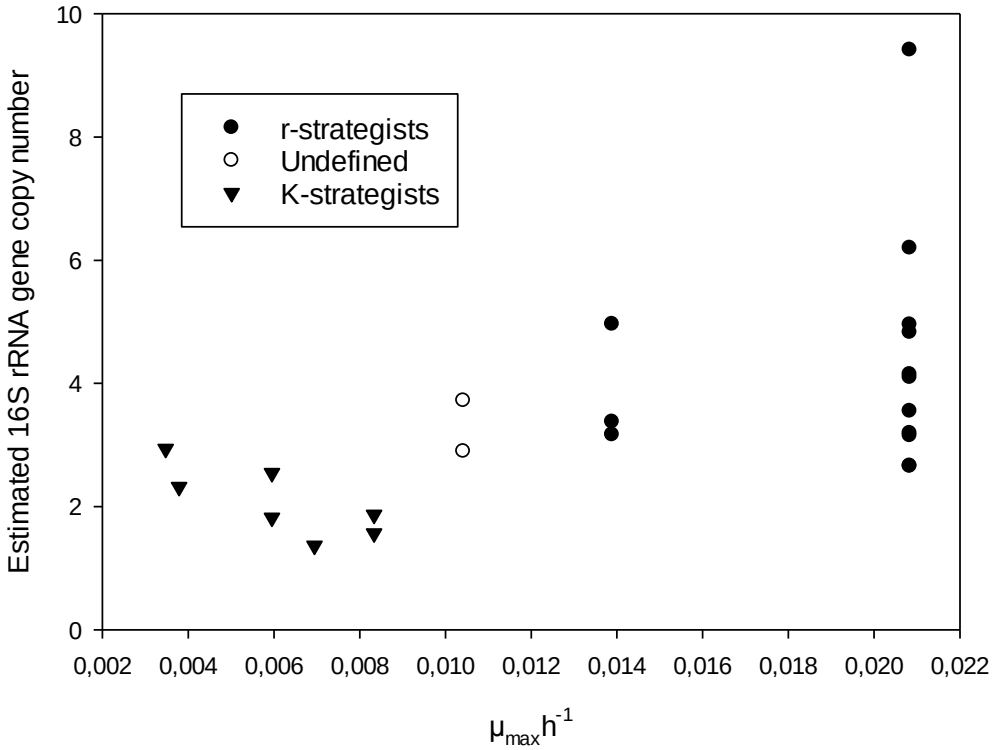

Figure 3.18: Estimated 16S rRNA gene copy number of the pure samples relative to maximum specific growth rate.

The slow-growing K-strategists generally were found to exhibit fewer 16S rRNA gene copy numbers per genome than the fast-growing r-strategists. The fast-growing r-strategists with the highest maximum specific growth rates showed large variation in 16S rRNA gene copy number. This might be related to the inaccurate determination of maximum specific growth rate.

The two reference strains *B.licheniformis* and *H.neptunium* had 16S rRNA gene copy numbers of 7 and 1 respectively. The genome size was relatively similar in both strains: 4 222 597 bp in *B.licheniformis* and 3 705 021 bp in *H.neptunium*. From the rtPCR analysis, the two strains were estimated to have around 4 copy numbers of the 16S rRNA gene in difference, whereas a variance of 6 copy numbers is the actual difference (Table 3.8). The reference strain *H.neptunium* had a broad and less uniform melting curve (Appendix N), indicating unspecific amplification, which might lead to an overestimation of the 16S rRNA gene copy number. Melting curves of *B.licheniformis* (Appendix N) and all of the isolated pure strains showed only one peak, indicating no unspecific amplification.

The amount of template DNA was used for normalizing the rtPCR results, due to a lack of primers for suitable one-copy reference gene. Different bacterial strains have differences in genome sizes. This will affect the normalization of the rtPCR results. If all genomes were equal in size, the normalization would give copy number of the 16S rRNA gene per genome. As the genome size varies, this might bias the 16S rRNA gene copy number estimation, because the ratio between total DNA concentration and target 16S rRNA concentration can differ in various strains. To investigate the correlation, genome size and 16S rRNA gene copy numbers were examined for 132 species for which genome sequences are published (Figure 3.19).

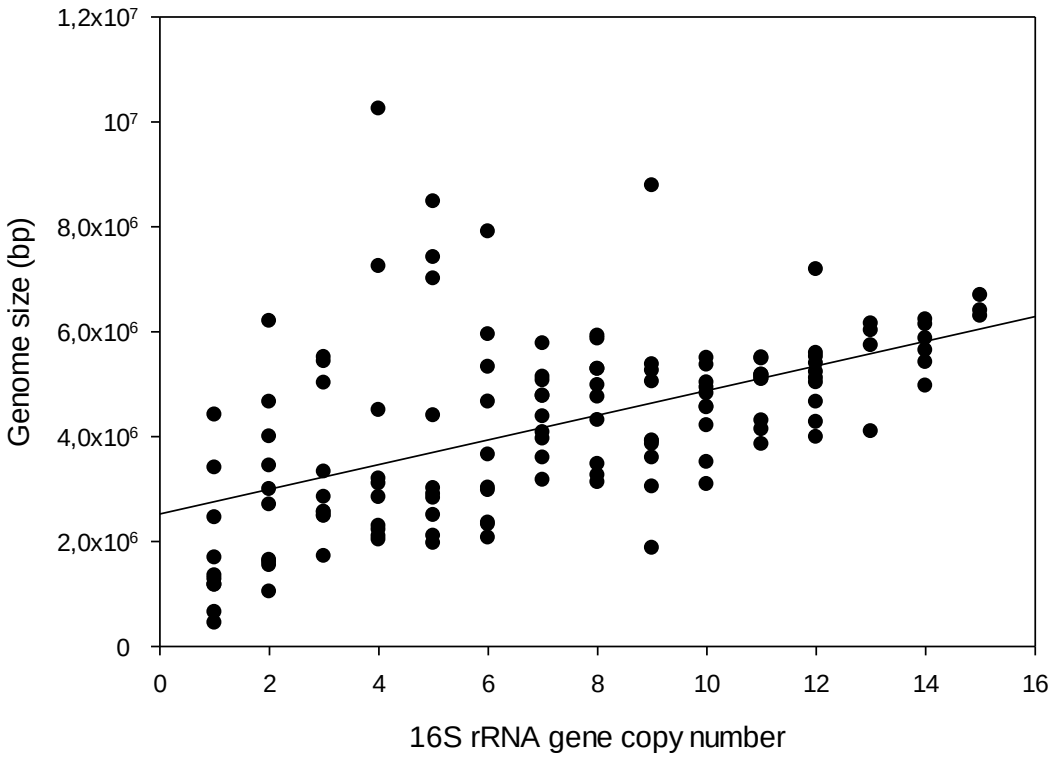

Figure 3.19: Copy number of 16S rRNA gene according to genome size. Data are gathered from 132 sequenced species from the Ribosomal RNA Database (Stoddard and Smith, 2015).

Most of the 132 species investigated has genome sizes between 2.0 * 10^6 and 5.0 * 10^6 (Figure 3.19). The general trend appears to be that the larger genome size, the more copy numbers of the 16S rRNA gene the microorganism exhibits. The effect of genome size could not be corrected for in the rtPCR analysis, as it was not known for any of the pure strains. The bias in genome size was thus disregarded in the analysis.

3.4.3 Estimation of 16S rRNA gene copy number in r- and K-selected communities

To estimate the average copy number of 16S rRNA gene per genome for r-and K-selected microbial communities, real-time PCR (rtPCR) analyses were performed on samples generated from seawater (surface and 90 meter) (see section 2.4.3).

The expected K-selected communities were hypothesized to have a lower average 16S rRNA gene copy number per genome, than the r-selected communities. Sampling from day 0-2, 17 and 24 was analyzed. The samples had been given a nutrient pulse at day 0, and a K-selection at day 0, succession towards r-selection at day 1-2 after nutrient imposed growth and a succession towards K-selection again at day 17-24 was expected. The 16S rRNA gene copy numbers were estimated (section 2.5.3.1) for the samples (Table 3.9 and Figure 3.20).

Table 3.9: rtPCR data for the surface (A) and the 90 meter seawater (B) communities at day 0, 1, 2, 17 and 24 of incubation. Mean Ct and mean amplification efficiency were based on the three replicates, with deviants excluded.

ID	Mean efficiency (E)	Mean Ct	E^{-Ct}	Estimated 16S rRNA gene copy number
A0	1.89	19.00	$5.71 \cdot 10^{-6}$	2.9
A1	1.88	17.19	$1.94 \cdot 10^{-5}$	9.7
A2	1.86	17.43	$2.03 \cdot 10^{-5}$	10.1
A17	1.90	19.29	$4.30 \cdot 10^{-6}$	2.1
A24	1.87	19.66	$4.35 \cdot 10^{-6}$	2.2
B0	1.87	20.98	$2.01 \cdot 10^{-6}$	1.0
B1	1.91	17.15	$1.49 \cdot 10^{-5}$	7.4
B2	1.97	16.16	$1.76 \cdot 10^{-5}$	8.8
B17	1.86	20.09	$4.07 \cdot 10^{-6}$	2.0
B24	1.83	19.46	$7.65 \cdot 10^{-6}$	3.8

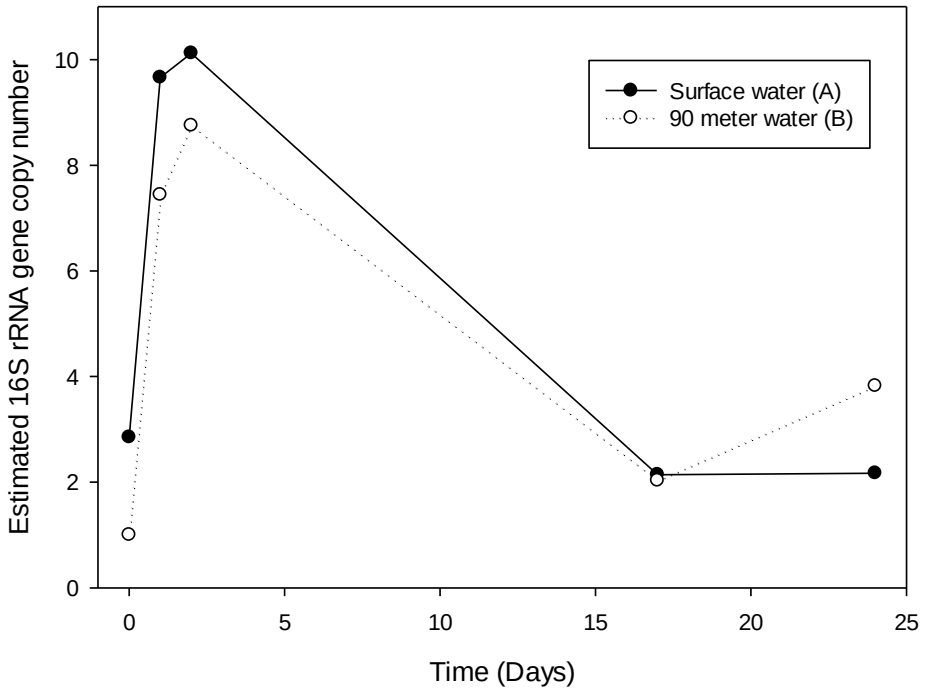

Figure 3.20: Estimated 16S rRNA gene copy number for the surface and 90 meter seawater communities according to time (days) after nutrient pulse on day 0.

At day 0 (K-selection) the estimated 16S rRNA gene copy numbers for the surface seawater sample was 1 copy per genome and around 3 for the 90 meter seawater sample (Figure 3.20). After an r-selection was induced by nutrient addition (initial shift) (see section 2.4.3), the copy number increased to around 8 and 10 copies per genome for the 90 meter and the surface seawater samples, respectively (Figure 3.20). When the nutrients were consumed and a succession towards K-selection occurred, the estimated copy number decreased to about the level found for the day 0 samples. This gave an average of 2.3 16S rRNA gene copy numbers per genome in K-selected communities and 9.0 in r-selected communities.

Melting curve analyses were performed for the rtPCR products obtained. The melting curves showed a broader and less discrete peak in communities for the samples that were assumed to represent K-selected communities (day 0, 17 and 24), thus indicating a higher diversity of 16S rDNA sequences with different melting points (Figure 3.21). In both communities, at day 1 and 2 where an r-selected pressure was expected, the melting curve showed only one peak. This indicates that a consequence of the growth of opportunistic r-strategists is a less diverse community.

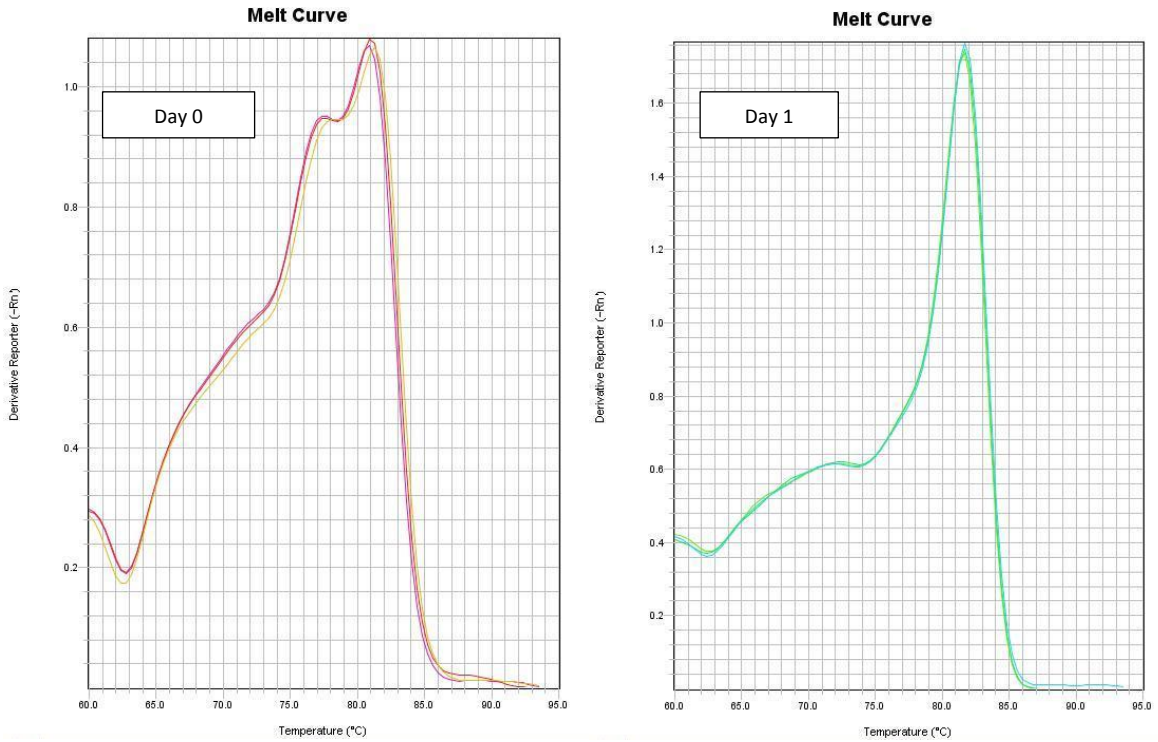

Figure 3.21: Melting curve for 16S rDNA amplicons from the 90 meters seawater at day 0 and day 1. Triplicate rtPCR reactions are shown. Derivative reporter (-Rn) is the change in fluorescent divided by change in temperature and is plotted against temperature.

3.5 Test of primers for amplification of the single-copy gene *cpn60* for possible digital droplet PCR analysis

For a more accurate 16S rRNA genome copy number determination by rtPCR, a single copy gene might be used for normalization. Other phylogenetic markers representing genes with only one copy in the genome, might be good candidates for normalization of the rtPCR results. These genes encodes proteins. The conserved regions are few and the third codon positions are highly variable (Kirchman, 2012). Universal bacterial primer pairs targeting these protein-coding genes contains degenerated sites or inosin, to anneal to and amplify the target DNA for all the bacterial strains in a microbial community. This creates the need for non-standard PCR conditions, like low annealing temperature. Due to the need for non-standard PCR conditions, such primers are thus not compatible with standard rtPCR analysis.

Digital droplet PCR (ddPCR) is an end-point quantitative PCR, which not depends on amplification efficiency, and could be more suitable to amplify protein-coding genes with degenerated primers. To test if the universal cpn60 primer pair cocktail (section 2.5.5) could be used to amplify a specific fragment of the *cpn60* gene, a traditional end-point PCR was run (Figure 3.22) with the PCR reaction mix from the ddPCR supplier (Bio-Rad Laboratories). DNA from two pure cultures (R1 and K23) were used as templates in PCR reactions with a standard annealing temperature of 60°C as recommended by the ddPCR supplier, both with the cpn60 primers and with the 16S rRNA primers as a control. The amplification product for the cpn60 primers was expected to be approximately 550 basepairs (bp). While the 16S rRNA primers gave a specific amplification product of expected length, the cpn60 primers resulted in no product at 60°C (Figure 3.22).

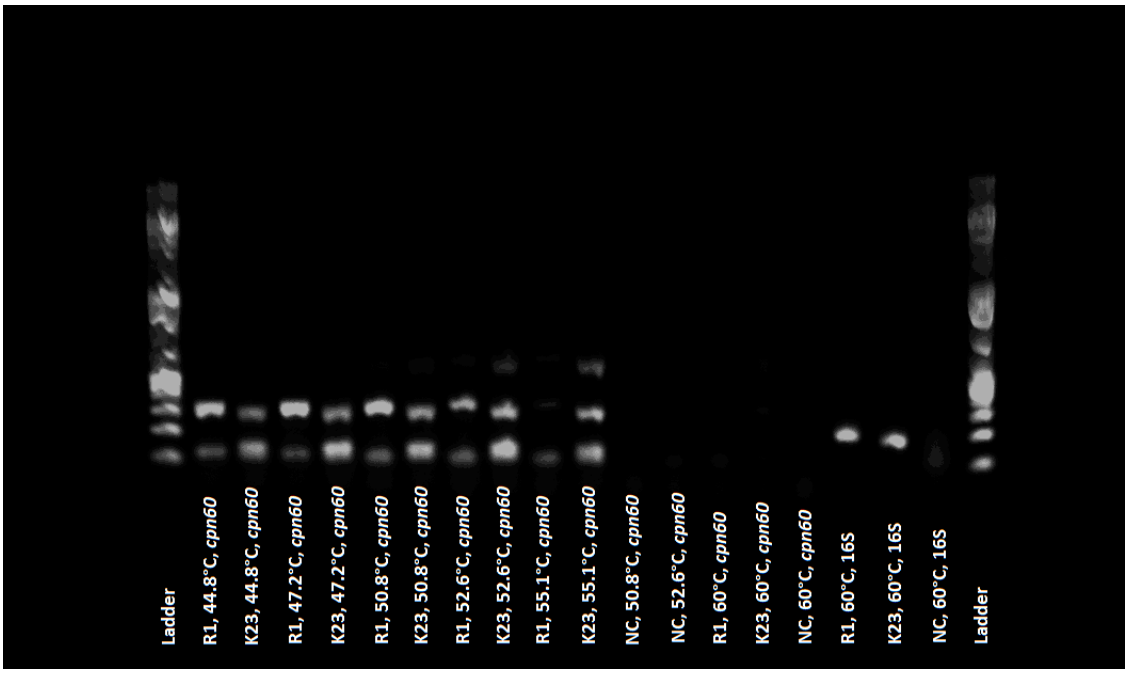

Figure 3.22: Gel electrophoresis with cpn60 and 16S rRNA PCR products for the R1 and K23 samples at different annealing temperatures. Annealing temperatures, template and target gene is indicated below the gel lanes. NC is the non-template control.

To investigate how different annealing temperatures (lower than 60°C) affect the amplification with the cpn60 primers, the two pure cultures R1 and K23 were amplified using 5 different annealing temperatures between 44.8 to 55.1°C. However, for all these annealing temperatures, the amplification resulted in 2 and 3 products, and a specific amplification of the 550 bp product was not achieved (Figure 3.22). Therefore, the cpn60 primers were not well suited for ddPCR analysis.

4. Discussion

4.1 Maximum RNA content of single cells determined by flow cytometry as a tool for quantifying the fraction of r- and K-strategists

Pure cultures were isolated and added nutrition to reach μ_{max} in order to correlate maximum specific growth rate (μ_{max}) and maximum cellular RNA content of bacterial strains. Flow cytometry was evaluated as a candidate for classification of the fraction of r-or K-strategist and relied on maximum specific growth rate (μ_{max}) as an indicator. K-strategists have a low μ_{max} and r-strategists have a high μ_{max}. It's important to remember that growth rate and RNA content changes, based on the metabolic state of the cell. In microbial communities, not all active organisms are growing, but all growing organisms are active (Blazewicz *et al.*, 2013). With a nutrient pulse, it could theoretically be possible to induce μ_{max} of all members in the community, and thus also maximum RNA content per cell achieved. Because of the need for nutrient pulsing, this strategy is partly cultivation-based. As a community consists of different members with different requirements for obtaining maximum specific growth rates, it's not possible to get a perfect maximum RNA content of all the cells. The goal was to trigger as many of the members as possible to achieve the highest RNA content. The hypothesis was that the higher cellular RNA content (FL1 intensity) per cell, the higher fraction of r-strategists was present in the community.

4.1.1 Pure cultures of r- and K-strategists

That maximum RNA content of a cell and μ_{max} is correlated was confirmed in the experiment. This has been well documented also previously (Morse and Carter, 1949, Gale and Folkes, 1953, Schaechter *et al.*, 1958). The slow-growing K-strategist had a lower maximum cellular RNA content than the fast-growing r-strategist (Figure 3.6). The relationship between μ_{max} and ratio of cellular maximum/minimum RNA content for pure strains were fairly linear, with a couple of exceptions. The pure strains with the highest maximum specific growth rate showed variable maximum/minimum cellular RNA ratios (Figure 3.7). This could have been caused by the fact that all of these strains were counted at day 2 on the agar plates. The maximum specific growth rate was not precisely determined, as the counting of colonies was performed only every 24 hours. Some of these strains may thus have a higher μ_{max} than

others, even if they were registered with identical μ_{max}. Also, some exceptions are registered in the literature, like for *Synechococcus* and *Prochlorococcus* strains, which has no increase or a decrease in RNA content at the highest growth rates (Binder and Liu, 1998, Worden and Binder, 2003). This could also be the case for some of the pure strains examined.

Some of the strains that were classified within the same genus had different maximum specific growth rate. There are several possible explanations for this. Within a genus, there can be different species and strains with variable maximum specific growth rates (Hopkins *et al.*, 1998, Salvadó *et al.*, 2011). Moreover, the plating was performed directly from surface seawater and from surface seawater that had been given a nutrient pulse. The unpulsed surface seawater also contains fast-growing r-strategists that might have needed longer time to reach μ_{max}, due to a lag phase. The r-strategists in the pulsed seawater had overcome this lag phase during the pulsing period, and therefore, had no lag phase on the agar plates. Salvesen and Vadstein (2000) also experienced that the growth rate was higher (17±9 hours) for cells in the stationary growth phase.

The two populations of cells that was observed when analyzing pure strains representing K-strategists by flow cytometry after 4 and 8 hours of nutrient pulsing, can be caused by either resting cells or the presence of dead cells (Figure 3.5). Eilers *et al.* (2000) concluded that rapidly enriched cultivable bacteria like *Vibrio*, can react rapidly to changes in growth conditions, even if the cells are starved and have been through a long period of non-growth. This can explain why two populations were not seen in the flow cytometry analysis of the fast-growing r-strategic strains. In specific nutrient-limiting conditions, decreased RNA content is associated with decreased growth for some organisms (Mandelstam and Halvorson, 1960, Davis *et al.*, 1986, Tolker-Nielsen *et al.*, 1997). The nutrient limitations that occurred on the agar plates when colonies were growing larger, may have generated a decrease in cellular RNA content. The results obtained here (Table 3.6) indicate that the slow-growing K-strategist did not have the ability to rapidly react to changes in nutrient composition, but had a longer lag phase before the RNA content of the cells in the population increased. This was indicated by the existence of two populations of cells in flow cytometry analysis for the slow-growing K-strategist, but not for the fast-growing r-strategists within 8 hours of incubation with medium. The two cell populations could also be caused by contamination of the samples. Contamination was probably not the cause here, as the strains were investigated by DNA sequencing after reaching the stationary phase. For all strains, sequences were unique and

indicated pure strains for all the samples where two cell populations were seen in the flow cytometry analysis. When excluding these suspected dead/resting cells from the analysis, there was an increase in the mean cellular RNA content, compared to the mean RNA content when these cells were not excluded (Table 3.6).

It was assumed that the fast-growing r-strategist would divide sooner than the K-strategist, leading to a change in the composition of the community. Therefore, when investigating the maximal cellular RNA content within a community, flow cytometry analysis after nutrient additions should be performed prior to cell division. The fast-growing r-strategists appeared to divide within 8 hours of incubation at 15°C, whereas the slower growing K-strategist did not (Figure 3.4). Sampling for cell count at 0 hours was not performed and the fast growing r-strategist might have divided earlier than 4 hours. The same trend was seen in the experiment where the effect of temperature and incubation time was determined (Table 3.4). Cell division of some of the members occurred after 4 hours at 22°C and after 8 hours at 12°C. A consequence of this observation is that it might be determined how r-selected a community is, by estimating the fraction of cells that divide after a short incubation. This should be investigated further with a higher frequency of samplings, including a sampling at 0 hours. Such a defined incubation time to distinguish r- and K-strategists has not been reported in the literature before.

4.1.2 Mixed microbial communities representing K- and r-selection

The hypothesis of a correlation between μ_{max} and maximum RNA content was supported by this study, and therefore, the methods were tested further for samples representing microbial communities. The development of the mean cellular RNA content in the surface and the 90 meter seawater was as assumed for the pulsed samples, with a low cellular RNA content at day 0 (K-selection), higher cellular RNA content after nutrient addition (day 1) and gradually a succession to K-selection to day 24 (Figure 3.9). High cellular RNA content indicated a high fraction of r-strategists present (r-selection) (day 1). When nutrients gradually became limiting, the cellular RNA content decreased, indicating that the K-strategist were able to compete for nutrients (K-selection). The r-strategists were then likely outcompeted, and most of them had probably gone into starvation or died, resulting in a decrease in the mean cellular RNA content of the community. The results for both the surface and the 90 meter seawater was as expected for most samples, but a few deviating values were observed for the surface

seawater at day 7 and 9 (Figure 3.9). These samples did not show the expected trend with a gradual decrease in RNA content. On day 7, there was a large decrease in cellular RNA content, which can be explained by dead r-strategists present after the shift (not lysed). As dead cells were counted, this may create a falsely low RNA content. This is supported by the fact that cell counts on day 7 were higher than for e.g. day 5 (Figure 3.8). At day 9, the mean RNA content increased above expected, compared to day 13. Possibly, some aggregated cells may have been included in the flow cytometry analysis, contributing to overestimation of cellular RNA content.

After the pulsing, the surface seawater had a higher RNA content at the start of the experiment than the 90 meter seawater, indicating that it initially had a less K-selected community (Figure 3.9). This was also hypothesized, because communities at the surface probably have access to more nutrients than microbial communities at 90 meters depth. Due to higher nutrient supply, the surface seawater probably had a higher proportion of fast-growing r-strategist who divided faster, compared to the 90 meter seawater with a more stable K-selected community. There was no increase in RNA content per cell after the pulsing (6 hours) of samples on day 1 in any of the communities, probably due to an excess of nutrients from the nutrient addition at day 0 (initial shift).

Because the cellular maximum RNA content is correlated with the maximum specific growth rate, the frequency distribution of RNA content among cells in a community could be a possible indicator of the percentage r-strategist or percentage K-strategist within a community. The distribution of RNA content within the communities over a time period of 24 days was determined. With the suggested fluorescent threshold, the K-strategic cells have a fluorescent intensity below 27 440, and the r-strategic cells have a fluorescent intensity over 53 782, when pulsed with nutrients and incubated for 6 hours (see section 3.3.4.1). The suggested fluorescent intensity threshold, were based on the assumption of the communities and evaluation of a threshold that maximizes the dynamic variability in the surface and 90 meter seawater over the 24 days of incubation. As some cells are neither r- nor K-selected species (27 440 and 53 782 in fluorescent intensity), these cells were not defined as either r- or K-strategists.

Based on the suggested thresholds, the percentage r-strategist or K-strategists were calculated (Table 3.7). As hypothesized, the surface seawater community had a higher fraction or r-

strategists than the 90 meter seawater from the start of the experiment. A high fraction of r-strategists were seen in both communities at day 1-3, indicating that the experimental r-selection was achieved. A succession to K-selection after the peak in cell densities was indicated by a gradual decrease in percentage r-strategists and an increase in percentage K-strategists towards day 24. However, a few exceptions were observed and include the deviating samples from the surface seawater at day 4, 5, 7 and 9. At day 4, 5 and 7 the percentage K-strategists were higher than assumed. This could have been caused by a large amount of dead r-strategists from the initial shift that was not lysed, and thus were counted as K-strategists, as the increase in RNA content towards a maximum was absent in dead cells (no metabolism). On day 9, there was an unexpected increase in percentage r-strategists (compared to day 3 and 13), which could be caused by clogging and non-optimal growth conditions, that could influence the RNA content determination with flow cytometry.

4.2 Relative 16S rRNA gene copy number determined by quantitative PCR as a tool for quantifying the fraction of r- and K-strategists

4.2.1 Pure cultures of r- and K-strategists

For the pure strains analyzed with real-time PCR (rtPCR), it was found a correlation between the estimated genomic 16S rRNA gene copy number and maximum specific growth rate (Figure 3.18). It can thus be stated that the slow-growing K-strategists exhibit fewer 16S rRNA gene copy numbers than the fast-growing r-strategists. This has been well documented also previously (Klappenbach *et al.*, 2000, Vieira-Silva and Rocha, 2010). The fast growing r-strategists with $\mu_{max}h^{-1}$ of 0.0208 (R1-R11) showed highly variable 16S rRNA gene copy numbers per genome (Figure 3.18), varying between 2.7 and 9.4 copies. With a more accurate determination of maximum specific growth rate ($\mu_{max)}$, an improved correlation between μ_{max} and 16S rRNA gene copy number could possibly be achieved.

The genomic 16S rRNA copy numbers were estimated relative to *B.licheniformis*, which has a known copy number of 7. The reference strain *H.neptunium* was estimated to have a genomic copy number of 2.9 of the 16S rRNA gene, while 1.0 were the actual copy number. The difference in copy numbers between the reference strains were thus 4 copies, whereas the

actual difference is 6 copy numbers. Amplification efficiency was corrected for and the strains had similar genome size. This demonstrates that there is a considerable uncertainty in the method. The melting curve for the 16S rDNA amplicon obtained for the reference strain *H.neptunium*, showed a broad peak and not a uniform curve, indicating unspecific amplification. This leads to an overestimation of the 16S rRNA gene copy number. For all the other pure strains and for the reference strain *B.licheniformis*, the rtPCR amplicons showed sharp melting curves, indicating a specific PCR product. Thus, overestimation of the genomic 16S rRNA copy number for these due unspecific amplification was probably not a problem.

4.2.2 Mixed microbial communities representing K- and r-selection

When applying the rtPCR method for samples of bacterial communities, a clear difference between the mean 16S rRNA gene copy number per genome was observed in the different samples. When a K-selected community was expected, the estimated 16S rRNA gene copy number was between 1.0 and 3.8 (Table 3.9) for the surface seawater samples and the 90 meter samples, respectively. In comparison, when an r-selected community was expected the estimated 16S rRNA gene copy number varied between 7.4 and 10.1 for the surface and the 90 meter seawater, respectively. Thus, in r-selected communities, the 16S rRNA gene copy number was considerably higher than for K-selected communities, in both the surface and the 90 meter seawater samples. An increase of approximately 7.5 16S rRNA gene copy number was observed in both seawater sample types.

The melting curves of PCR products of samples representing r-selected communities had a sharp and symmetric curve with one distinct peak (Figure 3.21). The melting curves of PCR products representing K-selected communities were broader and asymmetric, and in addition showed tendency of several peaks. The melting curve indicates that the PCR product consist of many different DNA sequences, which indicates a high diversity. This is interesting, as the communities were K-strategists dominate, are proposed to have a higher diversity (Vadstein *et al.*, 1993). It might be possible to use the melting curve as an indicator of diversity, and therefore also r/K-selection in microbial communities. Interestingly, Torsvik *et al.* (1996) investigated the melting profile and reassociation of 16S rDNA amplicons for sediment bacteria and soil bacteria. They proposed that the broad melting curve in the sediment community was an indication of a high diversity, compared to the soil, which had a sharper melting curve.

4.2.3 Real-time PCR normalization

The accuracy of the 16S rRNA gene copy number estimation was dependent on the exactness of the amount of template used in the rtPCR reactions for normalization. All the samples were analyzed on the same day, with the identical standards, to avoid variance in determination of concentration on a day to day basis. A disadvantage with the normalization of the rtPCR method was the variation in amplification efficiency among templates. This was however accounted for, by calculating the efficiency based on linear regression and correct for Ct value. The main problem with normalization according to DNA template concentrations is that there will be a difference in genome size among different species in a community. This is a source of bias in the analysis of mixed microbial communities (Farrelly *et al.*, 1995). The relative proportion of non-target DNA to target DNA is reduced when genome size is large (Garner, 2002). The proportion of target DNA in a given amount of template will thus be diluted, as the genome size increases. In addition, if the samples contain other organisms than bacteria, these will contribute to the isolated DNA concentration.

It was not possible to correct for genome size in the analysis in this study, because the genome size was not known for the isolated pure strains. Farrelly *et al.* (1995) investigated the effect genome size had on the amplification 16S rDNA fragments from a mixture of bacterial species and indicated that as long as genome size is unknown for the species present, it's impossible to quantify the numbers of species. The genome sizes within genus and even species are variable (Stoddard and Smith, 2015). An investigation of the genome size among 132 sequenced strains examined here showed a correlation between genome size and number of 16S rRNA gene copy numbers (Figure 3.19). This indicates that large genomes could lead to an underestimation of the 16S rRNA gene copy number determination, and that small genomes could lead to an overestimation. The 16S rRNA gene copy number is correlated with maximum specific growth rate, and it may be possible to correct for genome size in a theoretically manner, based on maximum specific growth rate. Further investigation is required to verify the statement.

The ideal would be to use gene with one copy per genome for normalization, because the aim was to determine the genomic 16S rRNA gene copy number. To use a single copy gene, the gene must be universally distributed, and there is a need for universal primers that targets the gene in all bacterial species. Because databases of single-copy genes this contains a small

number of sequenced data, it's a challenge to determine how conserved the candidate genes are among different taxa. Moreover, the single-copy genes often encode proteins. Conserved regions in protein genes are few and the third base of the nucleotide triplet that encodes amino acids are highly variable (Kirchman, 2012). This makes it challenging to design universal primers to amplify regions of the protein-coding gene in mixed microbial communities. The use of degenerated seats in the primer pairs used to amplify these protein coding genes makes them not well suited for rtPCR analysis, due to requirements of non-standard PCR conditions.

The digital droplet PCR (ddPCR) method was a proposed alternative to the rtPCR analysis. A single copy gene would be the ideal for normalization when estimating genomic 16S rRNA gene copy number. The aim was to include a single-copy protein gene into a ddPCR analysis. The ddPCR method has several advantages compared to the rtPCR method. It is both amplification efficiency independent, and has shown to be more sensitive and precise when determining low copy numbers (Sanders *et al.*, 2011, Hindson *et al.*, 2011). Primers for the Chaperon-60 gene (*cpn60*) were tested in a traditional PCR, but under the conditions typically used for ddPCR, and resulted in 1-2 unspecific PCR products at all the annealing temperatures tested (Figure 3.22). At the highest temperature (60°C), no PCR product was obtained. This annealing temperature was probably too high, due to inosine in some positions. Because the primers gave unspecific amplification and one specific PCR-product of the single-copy gene was not obtained, the primer pairs were thus not suited for ddPCR analysis.

4.3 Evaluation and comparison of methods

The flow cytometry method was shown to be a good strategy to determine the fraction of r- or K-strategist in seawater samples from mixed microbial communities. It can be used for the determination of either percentage r-strategists or percentage K-strategists, depending on the need. When determining the fraction of r-strategists, the highest cellular fluorescent intensities (RNA content) were used. For higher fluorescent signal strength, there is a possibility that aggregated cells were included in the counting, and this can give a falsely high estimate of RNA content. Most of the aggregated cells could be identified in the flow cytometry analysis and were excluded from the analysis, but there is no guaranty that no aggregates were present, due to the variable size of cells. When determining the K-strategists by identifying the cells with low maximum RNA content, the disadvantage could be that the slow-growing cells have been shown to adapt more slowly than the r-strategists to a nutrient pulse. Therefore, some

cells might not get a maximum increase in RNA content after nutrient pulse, because they are in a resting phase. Dead cells would not get an increase in RNA content, as no metabolism is present.

If the water samples to be analyzed are containing small clots or plaque (aggregated cells) the determination of r-strategists and the percentage K-strategists can be more precisely determined, because aggregated cells is not counted at the lowest fluorescent intensities. If there are no problems with aggregated cells in water samples to be analyzed, the determinations of percentage r-strategists are a safer choice, than determination of K-strategists, because dead and resting cells with low fluorescent intensities would not be counted. The properties of the water samples to be analyzed is therefore of importance when deciding to determine either the fraction of r- or K-strategists.

With the flow cytometry method there is a need to pulse the cells with nutrients and incubate them before maximum RNA content is achieved. The medium used (M65) is conducted to be the medium that gives the highest CFU (Colony-forming units) when cultivating seawater bacteria (Slaatebræk, 1975). Still, how to trigger maximum specific growth rate and rRNA synthesis in uncultivable cells is not known, as the optimal growth conditions are not identified. This is the most important disadvantage of the flow cytometry method, because the method is dependent on a nutrient pulse and incubation to induce maximum specific growth rate and rRNA synthesis. The flow cytometry analysis is thus only partly cultivation-independent.

For the suggested rtPCR method, it was not possible to determine the exact 16S rRNA gene copy number per genome, because it depends on a more ideal normalization. Because genome size was not accounted for, it can be argued that the rtPCR method is not accurate enough to determine the fraction of r-strategists or K-strategists in a mixed microbial community. This is especially the case if the samples contain other organisms that will contribute to the isolated DNA. Still, the estimated 16S rRNA gene copy numbers per genome in r-selected communities were considerably higher than for the K-selected communities. The method can give an indication of the diversity and degree of strategy within the community, by studying the estimated genomic 16S rRNA gene copy number and the melting curve.

Of the two method strategies evaluated, the most accurate one was the one based on flow cytometry. This was also the most cost- and time-efficient method. With the rtPCR method, there is a need to isolate DNA and precisely determine of DNA template concentrations. In addition, several reagents for the rtPCR are needed, creating a more expensive analysis. For the flow cytometry based analysis, the SYBR Green II stain is the only reagent needed, except nutrients for pulsing and the washing solutions for the flow cytometry. The time used for the analysis is considerably lower for the flow cytometry method. The two evaluated methods, flow cytometry and rtPCR, rely on analysis of RNA and DNA, respectively. Because a certain number of rRNA transcripts can be initiated from a single rRNA operon promoter (Klappenbach *et al.*, 2000), the higher copy number of the 16S rRNA gene, the more rRNA can potentially be produced. Hence, the synthesis of rRNA that is achieved after the pulsing of nutrients would likely be larger for cells with many copies of the 16S rRNA gene in their genome.

For a more precise determination of 16S rRNA gene copy number, a single-copy gene suitable for design of universal bacterial PCR primers should be found and used in the ddPCR analysis. The fraction of the r-strategists could be determined more precisely, and bias due to differences in genome sizes is avoided. As there is no need for cultivation or medium pulse, a quantitative PCR method like this would also detect uncultivable cells. It would thus be more accurate than the flow cytometry method, even if flow cytometry is more time- and cost-efficient.

4.4 Application of methods

The presented methods can have a wide range of applications. First of all, especially the flow cytometry based method can be used to determine the fraction of r- or K-strategist in rearing water in aquaculture system for marine fish larvae. Multiple publications have shown that a high biological control, with stable K-selection gives increased survival and growth of marine larvae (Vadstein *et al.*, 1993, Skjermo *et al.*, 1997, Skjermo and Vadstein, 1999, Attramadal *et al.*, 2012). The distribution of RNA content among cells in a community gives an indication of the distribution of maximum specific growth rate for the cells, or it can be used as an indication of the diversity within the community. The flow cytometry strategy described is less time-consuming than cultivation-dependent methods and creates the possibility to monitor the microbial environment continuously throughout the rearing period. Moreover, the

flow cytometry strategy can easy be adapted to use in fresh water samples, for example in rearing of Atlantic salmon. In addition, it can also be adapted to be used in ecological studies in natural ecosystems. With further modification, the flow cytometry method might also be used in determining the purity of drinking water, etc. Pathogens are in general r-strategists and determination of the r-strategists in drinking water can give an indication of contamination.

4.5 Further work

Further work for the flow cytometry based method is to optimize the growth conditions in the nutrient pulsing of mixed microbial communities. During cultivation of seawater with mixed microbial communities, aggregation of cells was observed. A rerun of the experiments under different growth conditions, like less nutrient concentration, temperature variations and oxygenation, can be possible actions to prevent the aggregation. The influence of the clogging on the flow cytometry results could thus be assessed. Moreover, it would be an advantage to determine a more precise incubation at different temperatures for the pure strains, for which cell division of r-strategic cells occur, and cell division of K-strategists do not. The frequency of sampling should be increased (within the 8 hour range), and include a "0 hour" sample, representing the number of cells prior to incubation. The flow cytometry strategy should also be tested with other seawater communities, to additionally support the statement that the method is suitable to determine the fraction of r- or K-strategist in rearing water. In additional, the similar experimental strategy tested in freshwater, with a suitable medium, can increase the area of application of the method.

For a more accurate genomic 16S rRNA gene copy number determination with quantitative-PCR, universal bacteria primers that target a single-copy gene should be designed. Suitable primers should then be tested for a potential ddPCR analysis, and thus the bias in genome size will be eliminated and a more precise normalization achieved. Then, the rtPCR or the ddPCR can be a better alternative than the flow cytometry strategy in the determination of r- or K-strategists. This is because quantitative PCR methods have one vast advantage, in contrast to the flow cytometry strategy: it also would include uncultivable cells for certain. If an optimal normalization of one of the quantitative PCR methods is achieved, the method would have the same application as the flow cytometry.

5. Conclusion

The investigation of pure microbial strains and mixed microbial communities confirmed the theory: r-strategist with a high maximum specific growth rate has a higher maximum cellular RNA content and a high number of 16S rRNA gene copy numbers in their genome, compared to the slow-growing K-strategist.

Of the two methods tested, the flow cytometry strategy is best suited to determine the fraction of r- or K-strategists in seawater. The method can be used to determine the frequency distribution of maximum specific growth rate among cells within mixed microbial communities, and may therefore be used to determine the fraction of r- or K-strategists in seawater, e.g. rearing water for marine fish larvae. The flow cytometry method is cost-efficient and the time for analysis is low. The method can easily be adapted to fresh water samples to extend the area of application. However, the flow cytometry method is based on pulsing with nutrients to induce maximum cellular RNA content. This is a disadvantage, as we do not know the optimal growth conditions for uncultivable cells.

For the rtPCR method, an accurate estimate of the 16S rRNA gene copy number per genome was not achieved, due to the lack of a precise normalization of the rtPCR results. The fraction of r- and K-strategist within microbial communities could thus not be determined in a precise manner. Ideally, normalization could be achieved by using a single-copy protein gene. To do this, universal bacterial PCR primers targeting a single-copy protein gene have to be designed. If a successful amplification of a single-copy gene with universal bacterial primers can be achieved, either one of the quantitative-PCR methods (rtPCR/ddPCR) can be an alternative to the flow cytometry strategy in the determination of r- or K-strategists. A method like this would be cultivation-independent, as opposed to the flow cytometry method.

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List of appendices

Appendix A: Cultivation medium used in the project

Table A.1: M65 agar with a concentration of 0.5 g/l of each component (yeast extract, peptone, tryptone). The M65 agar was used for cultivation of pure cultures from surface seawater.

Component	Amount	Supplier	Kat.nr/LOT
Yeast extract	0.5 g	Oxoid LTD.	LP0021/1214988-02
Bacteriological Peptone	0.5 g	Oxoid LTD.	LP0037/1432643
Tryptone	0.5 g	Oxoid LTD.	LP0042/450463
Filtered seawater	800 ml		
Distilled water	200 ml		
Agar Bacteriological (Agar no.1)	15 g	Oxoid LTD.	LP0011/1285252-02

Table A.2: M65 liquid medium with a concentration of 0.5 g/l of each component (yeast extract, peptome, tryptone). The medium was used for cultivation of the pure isolated strains.

Component	Amount	Supplier	Kat.nr/LOT
Yeast extract	0.5 g	Oxoid LTD.	LP0021/1214988-02
Bacteriological Peptone	0.5 g	Oxoid LTD.	LP0037/1432643
Tryptone	0.5 g	Oxoid LTD.	LP0042/450463
Filtered seawater	800 ml	Oxoid LTD.	
Distilled water	200 ml	Oxoid LTD.	

Table A.3: M65 concentrated stock medium with a concentration of 50 g/l of each component (yeast extract, peptome, tryptone). 150 g/l in total, of the three components. Used in experiments for pulsing of seawater.

Component	Amount	Supplier	Kat.nr/LOT
Yeast extract	5 g	Oxoid LTD.	LP0021/1214988-02
Bacteriological Peptone	5 g	Oxoid LTD.	LP0037/1432643
Tryptone	5 g	Oxoid LTD.	LP0042/450463
Distilled water	100 ml	Oxoid LTD.	

Table A.4: Components of the Marine Broth agar used for cultivation of *Hyphomonas neptunium.* For Marine broth medium, all components except the agar was used.

Component	Amount	Supplier	Kat nr/LOT
Marine Broth	37.4 g	Difco ™	279110/3330098
Distilled water	1000 ml		
Agar Bacteriological (Agar no.1)	15 g	Oxoid LTD.	LP0011/1285252-02

Table A.5: Components of the Nutrient Broth agar used for cultivation of *Bacillus licheniformis.* For Nutrient Broth medium, all components except the agar was used.

Component	Amount	Supplier	Kat nr/LOT
Nutrient Broth	8.0 g	Difco ™	234000/4155935
Distilled water	1000 ml		
Agar Bacteriological (Agar no.1)	15 g	Oxoid LTD.	LP0011/1285252-02

Appendix B: f/2 medium

f/2 medium

f/2 medium (Guillard, 1975) is a general enriched seawater medium designed for graving marine algae, especially diatoms. Add the following components (Table B.1) into 950 ml of filtered natural seawater. Bring the final volume to 1 liter with filtered natural seawater and autoclave. For addition in seawater, 100 µl/l was used.

Table B.1: Components of the f/2 medium with concentration of each component in the medium and final concentration in the seawater.

Component	Stock solution (g/l)	Quantity used	Concentration in final medium (µg/ml)	Final concentration in seawater (µg/ml)
$NaNO_3$	75.0	1 ml	75.0	7.5
$NaH_2PO_4 \cdot H_2O$	5.0	1 ml	5.0	0.5
$Na_2SiO_3 \cdot 9H_2O$	30.0	1 ml	30.0	3.0
Trace metal solution	(See Table B.2)	1 ml	-	-
Vitamins solution	(See Table B.3)	0.5 ml	-	-

f/2 Trace Metals Solution

In 950 ml of dH_2O, dissolve the EDTA and the other components. Bring the final volume to 1 liter with dH_2O.

Table B.2: Components of the Trace Metals Solutions with concentration of each component in the medium and the final concentration in the seawater.

Component	Stock solution (g/l)	Quantity used	Concentration in final medium (µg/ml)	Final concentration in seawater (µg/ml)
$FeCl_3 \cdot 6H_2O$	-	3.15 g	3150.0	315.0
$Na_2EDTA \cdot 2H_2O$	-	4.36 g	4360.0	436.0
$MnCl_2 \cdot 4H_2O$	180.0	1 ml	180.0	18.0
$ZnSO_4 \cdot 7H_2O$	22.0	1 ml	22.0	2.2
$CoCl_2 \cdot 6H_2O$	10.0	1 ml	10.0	1.0
$CuSO_4 \cdot 5H_2O$	9.8	1 ml	9.8	0.98
$Na_2MoO_4 \cdot 2H_2O$	6.3	1 ml	6.3	0.63

f/2 Vitamin Solution

Into 950 ml dH_2O, dissolve the thiamine · HCl, and add 1 ml of the primary stocks. Bring final volume to 1 liter with dH_2O. Filter-sterilize and store frozen.

Table B.3: Components of the Vitamin Solution with concentration of each component in the medium and the final concentration in the seawater.

Component	**Stock solution (g/l)**	**Quantity used**	**Concentration in final medium (µg/ml)**	**Final concentration in seawater (µg/ml)**
Thiamine · HCl (vit.B)	-	200 mg	200	20.0
Biotin (vit.H)	1.0	1 ml	1.0	0.1
Cyanocobalamin (vit.B_{12})	1.0	1 ml	1.0	0.1

Appendix C: SYBR®Green II product sheet

Molecular Probes

Product Information

Revised: 03–April–2001

SYBR® Green II RNA Gel Stain

Quick Facts

Storage upon receipt:
- –20°C
- Desiccate
- Protect from light

Ex/Em: 254, 497/520 nm, bound to nucleic acid

Introduction

Molecular Probes' SYBR® Green II RNA gel stain is one of the most sensitive dyes known for detecting RNA in electrophoretic gels. As little as 100 pg RNA or single-stranded DNA per band can be detected in a SYBR Green II–stained agarose or polyacrylamide gel using 254 nm epi-illumination Polaroid® 667, black-and-white print film and a SYBR Green gel stain photographic filter (S-7569).[1,2] Even with 300 nm transillumination, as little as 500 pg RNA per band can be detected. SYBR Green II RNA gel stain is significantly more sensitive than ethidium bromide, the most commonly used stain for detecting nucleic acids in gels (Figure 1). With 300 nm transillumination and photography through an orange-red gelatin filter, ethidium bromide's sensitivity limit in a standard agarose minigel is about 1.5 ng single-stranded nucleic acid per band. Note that our detection limits are based on results obtained with a FotoDyne® Foto/UV® 450 ultraviolet transilluminator in combination with Polaroid 667 film. Video cameras and CCD cameras in general have a different spectral response than black-and-white print film and thus may not exhibit the same sensitivity.

On denaturing agarose/formaldehyde gels and polyacrylamide/urea gels, the sensitivity of SYBR Green II RNA gel stain is somewhat reduced, though still superior to that of ethidium bromide. To achieve maximal sensitivity with ethidium bromide, agarose/formaldehyde gels must be washed for several hours prior to staining. In contrast, *without any washing or destaining steps*, we have been able to detect 1 ng RNA per band in a SYBR Green II dye-stained agarose/formaldehyde gel or polyacrylamide/urea gel using 254 nm epi-illumination, and about 4 ng RNA per band using 300 nm transillumination.

The remarkable sensitivity of SYBR Green II RNA gel stain for detecting RNA can be attributed to several factors, including superior fluorescence quantum yield, binding affinity and fluorescence enhancement. Although it is not selective for RNA staining, this dye exhibits a higher quantum yield when bound to RNA (~0.54) than to double-stranded DNA (~0.36). This property is somewhat unusual among nucleic acid stains; most show far greater quantum yields and fluorescence enhancements when bound to double-stranded nucleic acids. The fluorescence quantum yield of the RNA/SYBR Green II complex is more than seven times greater than that of the RNA/ethidium bromide complex (~0.07).[3] The affinity of SYBR Green II RNA gel stain for RNA is also higher than that of ethidium bromide, and its fluorescence enhancement upon binding RNA is well over an order of magnitude greater. Because SYBR Green II RNA gel stain has a low intrinsic fluorescence, there is no need to destain gels to remove free dye. The fluorescence of RNA/SYBR Green II complexes is not quenched in the presence of urea or formaldehyde, eliminating the need to wash these denaturants out of gels prior to staining. In addition, staining agarose/formaldehyde gels with SYBR Green II RNA gel stain does not interfere with transfer of RNA to filters or subsequent hybridization in Northern blot analysis as long as 0.1%–0.3% SDS is included in prehybridization and hybridization buffers.

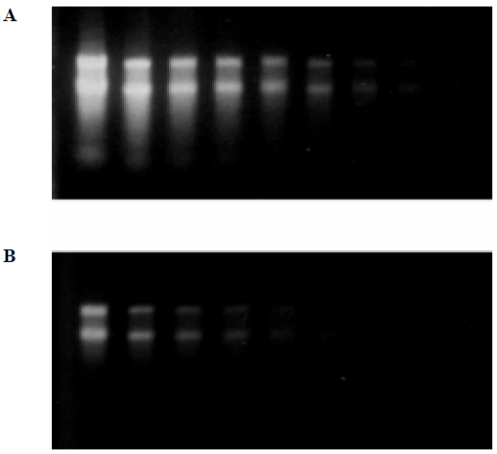

Figure 1. *Dilution series of Escherichia coli ribosomal 16S and 23S RNA electrophoresed in 1% agarose gels. The gels contain an identical twofold dilution series of RNA. The gel shown in panel A was stained for 20 minutes with SYBR Green II RNA gel stain (using a 1:10,000 dilution of the stock reagent) and not destained. The gel shown in panel B was stained for 20 minutes with 5 µg/mL ethidium bromide, then destained for an additional 20 minutes. The SYBR Green II dye-stained gel was excited using 254 nm epi-illumination and the ethidium bromide–stained gel using 300 nm transillumination (Fotodyne® Foto UV 450 ultraviolet transilluminator). Although the SYBR Green II dye-stained gel can be excited at 300 nm, epi-illumination at 254 nm resulted in the best sensitivity in our hands. Both gels were photographed with Polaroid 667 black-and-white print film, using a SYBR Green gel stain photographic filter (SYBR Green II dye-stained gel) or an ethidium bromide gel stain photographic filter (ethidium bromide–stained gel).*

SYBR Green II RNA gel stain can be used to:

- Analyze small aliquots from RNA preparations before Northern blotting, start-site mapping or cDNA preparation
- Visualize the migration behavior of 5S rRNA species after high-resolution denaturing gradient electrophoresis (DGGE)[4]
- Stain DNA in single-strand conformation polymorphism (SSCP)[5]
- Stain DNA before amplification by PCR[6]

Materials

Contents

SYBR Green II stain is provided as a 10,000X concentrate in DMSO in the following sizes:

- 500 µL (S-7564), sufficient to stain ~100 minigels
- 1 µL (S-7568), sufficient to stain ~50 minigels
- 20 vials (S-7586), each containing 50 µL

We also offer SYBR Green II stain the SYBR Green Nucleic Acid Gel Stain Starter Kit (S-7580), which includes:

- 50 µL SYBR Green I stain
- 50 µL SYBR Green II stain
- SYBR Green/Gold gel stain photographic filter

Storage

Upon receipt, store the dye frozen at -20°C, protected from light in a desiccator. When stored properly, the SYBR Green II stain stock solution in DMSO is stable for six months to one year.

Handling and Disposal

Before opening, each vial should be allowed to warm to room temperature and then briefly centrifuged in a microfuge to deposit the DMSO solution at the bottom of the vial.

We must caution that no data are available addressing the mutagenicity or toxicity of SYBR Green II RNA gel stain. Because this reagent binds to nucleic acids, it should be treated as a potential mutagen and used with appropriate care. The DMSO stock solution should be handled with particular caution as DMSO is known to facilitate the entry of organic molecules into tissues. We strongly recommend using double gloves when handling the DMSO stock solution. As with all nucleic acid stains, solutions of SYBR Green II RNA gel stain should be poured through activated charcoal before disposal. The charcoal must then be incinerated to destroy the dye.

Spectral Characteristics

SYBR Green II RNA gel stain may be used with commonly available ultraviolet epi- and transilluminator excitation sources, as well as hand-held ultraviolet lamps. SYBR Green II RNA gel stain is maximally excited at 497 nm, but also has a secondary excitation peak centered near 254 nm. The fluorescence emission of SYBR Green II dye-stained RNA is centered at 520 nm. These spectral characteristics make SYBR Green II RNA gel stain compatible with a wide variety of gel reading instruments, ranging from those with ultraviolet epi- and transillumination to argon-ion laser and mercury-arc lamp excitation gel scanners.

Experimental Protocols

Staining RNA Following Electrophoresis

1.1 Perform electrophoresis on nondenaturing gels or on denaturing polyacrylamide/urea or agarose/formaldehyde gels according to standard techniques.[7] SYBR Green II RNA gel stain has not been tested with other gel matrices.

1.2 Dilute the stock SYBR Green II RNA gel stain. For nondenaturing gels and denaturing polyacrylamide/urea gels, we recommend a 1:10,000 dilution in TBE (89 mM Tris base, 89 mM boric acid, 1 mM EDTA, pH 8). For denaturing agarose/formaldehyde gels, we recommend a 1:5000 dilution in TBE. Staining with SYBR Green II reagent is pH sensitive. For optimal sensitivity, verify that the pH of the staining solution at the temperature used for staining is between 7.5 and 8.0 (preferably pH 8.0).

1.3 Place the gel in a staining container, such as a Petri dish or the top of a pipet-tip box. Add enough staining solution to cover the gel. Protect the staining container from light by covering it with aluminum foil or placing it in the dark. There is no need to wash urea or formaldehyde out of gels prior to staining.

1.4 Agitate the gel gently at room temperature. The optimal staining time is typically 10–40 minutes for polyacrylamide gels and 20–40 minutes for agarose gels. The staining time may vary depending on the thickness of the gel and the percentage of agarose or polyacrylamide. No destaining is required. The staining solution may be stored in the dark (preferably refrigerated) and reused three to four times.

Visualizing and Photographing Stained Gels

2.1 Illuminate the stained gel using 300 nm ultraviolet transillumination, or for greater sensitivity, 254 nm epi-illumination.

2.2 For optimal sensitivity with black and white film, SYBR Green II–stained gels should be photographed through Molecular Probes' SYBR Green gel stain photographic filter (S-7569). A number of other yellow or green gelatin or cellophane filters (available from Kodak™ through photography equipment suppliers) can also be used for photography, but most will provide slightly reduced sensitivity. The orange-red filters used to photograph ethidium bromide–stained gels should not be used with SYBR Green II–stained gels.

2.3 Photograph the gel with Polaroid 667 black-and-white print film using a SYBR Green gel stain photographic filter. Stained gels have negligible background fluorescence, allowing long film exposures when detecting small amounts of RNA. For 300 nm transillumination, typically a 1–2 second exposure using an F-stop of 4.5 is adequate. For 254 nm epi-illumination (especially with a hand-held lamp), exposures on the order of 1–1.5 minutes may be required for maximal sensitivity.

Appendix D: Power Soil® DNA isolation kit

Experienced User Protocol

Please wear gloves at all times

1. To the **PowerBead Tubes** provided, add 0.25 grams of soil sample.
2. Gently vortex to mix.
3. **Check Solution C1.** If **Solution C1** is precipitated, heat solution to 60°C until dissolved before use.
4. Add 60 µl of **Solution C1** and invert several times or vortex briefly.
5. Secure **PowerBead Tubes** horizontally using the MO BIO Vortex Adapter tube holder for the vortex (MO BIO Catalog# 13000-V1-24) or secure tubes horizontally on a flat-bed vortex pad with tape. Vortex at maximum speed for 10 minutes.
   Note: If you are using the 24 place Vortex Adapter for more than 12 preps, increase the vortex time by 5-10 minutes.
6. Make sure the PowerBead Tubes rotate freely in your centrifuge without rubbing. Centrifuge tubes at 10,000 x *g* for 30 seconds at room temperature. **CAUTION:** Be sure not to exceed 10,000 x *g* or tubes may break.
7. Transfer the supernatant to a clean **2 ml Collection Tube** (provided).
   Note: Expect between 400 to 500 µl of supernatant. Supernatant may still contain some soil particles.
8. Add 250 µl of **Solution C2** and vortex for 5 seconds. Incubate at 4°C for 5 minutes.
9. Centrifuge the tubes at room temperature for 1 minute at 10,000 x *g*.
10. Avoiding the pellet, transfer up to, but no more than, 600 µl of supernatant to a clean **2 ml Collection Tube** (provided).
11. Add 200 µl of **Solution C3** and vortex briefly. Incubate at 4°C for 5 minutes.
12. Centrifuge the tubes at room temperature for 1 minute at 10,000 x *g*.
13. Avoiding the pellet, transfer up to, but no more than, 750 µl of supernatant into a clean **2 ml Collection Tube** (provided).
14. Shake to mix Solution C4 before use. Add 1200 µl of **Solution C4** to the supernatant and vortex for 5 seconds.
15. Load approximately 675 µl onto a **Spin Filter** and centrifuge at 10,000 x *g* for 1 minute at room temperature. Discard the flow through and add an additional 675 µl of supernatant to the **Spin Filter** and centrifuge at 10,000 x *g* for 1 minute at room temperature. Load the remaining supernatant onto the **Spin Filter** and centrifuge at 10,000 x *g* for 1 minute at room temperature.
    Note: A total of three loads for each sample processed are required.
16. Add 500 µl of **Solution C5** and centrifuge at room temperature for 30 seconds at 10,000 x *g*.
17. Discard the flow through.
18. Centrifuge again at room temperature for 1 minute at 10,000 x *g*.
19. Carefully place spin filter in a clean **2 ml Collection Tube** (provided). Avoid splashing any **Solution C5** onto the **Spin Filter**.
20. Add 100 µl of **Solution C6** to the center of the white filter membrane. Alternatively, sterile DNA-Free PCR Grade Water may be used for elution from the silica Spin Filter membrane at this step (MO BIO Catalog# 17000-10).
21. Centrifuge at room temperature for 30 seconds at 10,000 x *g*.
22. Discard the **Spin Filter**. The DNA in the tube is now ready for any downstream application. No further steps are required.

We recommend storing DNA frozen (-20° to -80°C). **Solution C6** contains no EDTA. To concentrate the DNA see the Hints & Troubleshooting Guide.

Thank you for choosing the PowerSoil® DNA Isolation Kit.

Appendix E: Qubit™ dsDNA HS assay kit

Handling and Disposal

We must caution that no data are available addressing the mutagenicity or toxicity of the Qubit™ dsDNA HS reagent (Component A). This reagent is known to bind nucleic acid and is provided as a solution in DMSO. Treat the Qubit™ dsDNA HS reagent with the same safety precautions as all other potential mutagens and dispose of the dye in accordance with local regulations.

Experimental Protocol

The protocol below assumes you will be preparing standards for calibrating the Qubit® 2.0 Fluorometer. If you plan to use the last calibration performed on the instrument, you will need fewer tubes (step 1.1) and less working solution (step 1.3). More detailed instructions on the use of the Qubit® 2.0 Fluorometer (corresponding to steps 1.9–1.15 and 2.1–2.6) can be found in the user manual accompanying the instrument.

1.1 Set up the number of 0.5 mL tubes you will need for standards and samples. The Qubit™ dsDNA HS assay requires 2 standards.

Note: Use only thin-wall, clear 0.5 mL PCR tubes. Acceptable tubes include Qubit™ assay tubes (500 tubes, Cat. no. Q32856) or Axygen PCR-05-C tubes (VWR, part no. 10011-830).

1.2 Label the tube lids.

1.3 Make the Qubit™ working solution by diluting the Qubit™ dsDNA HS reagent 1:200 in Qubit™ dsDNA HS buffer. Use a clean plastic tube each time you make Qubit™ working solution. Do not mix the working solution in a glass container.

Note: The final volume in each tube must be 200 µL. Each standard tube will require 190 µL of Qubit™ working solution, and each sample tube will require anywhere from 180 µL to 199 µL. Prepare sufficient Qubit™ working solution to accommodate all standards and samples. For example, for 8 samples, prepare enough working solution for the samples and 2 standards: ~200 µL per tube in 10 tubes yields 2 mL of working solution (10 µL of Qubit™ reagent plus 1,990 µL of Qubit™ buffer).

1.4 Load 190 µL of Qubit™ working solution into each of the tubes used for standards.

1.5 Add 10 µL of each Qubit™ standard to the appropriate tube and mix by vortexing 2–3 seconds, being careful not to create bubbles.

Note: Careful pipetting is critical to ensure that exactly 10 µL of each Qubit™ dsDNA HS standard is added to 190 µL of Qubit™ working solution. It is also important to label the lid of each standard tube correctly as calibration of the Qubit® 2.0 Fluorometer requires that the standards be introduced to the instrument in the right order.

1.6 Load Qubit™ working solution into individual assay tubes so that the final volume in each tube after adding sample is 200 µL.

Note: Your sample can be anywhere between 1 µL and 20 µL, therefore, load each assay tube with a volume of Qubit™ working solution anywhere between 180 µL and 199 µL.

1.7 Add each of your samples to assay tubes containing the correct volume of Qubit™ working solution (prepared in step 1.6) and mix by vortexing 2–3 seconds. The final volume in each tube should be 200 µL.

1.8 Allow all tubes to incubate at room temperature for 2 minutes.

1.9 On the Home Screen of the Qubit® 2.0 Fluorometer, press **DNA**, and then select **dsDNA High Sensitivity** as the assay type. The Standards Screen is automatically displayed.

Note: If you have already performed a calibration for the selected assay, Qubit® 2.0 Fluorometer will prompt you to choose between reading new standards and using the previous calibration. See *Calibrating the Qubit® 2.0 Fluorometer* above for calibration guidelines.

1.10 On the Standards Screen, press **Yes** to run a new calibration or press **No** to use the last calibration.

1.11 If you pressed **No** on the Standards Screen, proceed to step 1.12. If you selected **Yes** to a run new calibration, follow instructions below.

Running a New Calibration

Insert the tube containing Standard #1 in the Qubit® 2.0 Fluorometer, close the lid, and press **Read**. The reading will take approximately 3 seconds.

Remove Standard #1.

Insert the tube containing Standard #2 in the Qubit® 2.0 Fluorometer, close the lid, and press **Read**.

Remove Standard #2.

1.12 If you pressed **No** on the Standards Screen, the Sample Screen will be automatically displayed. Insert a sample tube into the Qubit® 2.0 Fluorometer, close the lid, and press **Read**.

1.13 Upon the completion of the measurement, the result will be displayed on the screen.

Note: The value given by the Qubit® 2.0 Fluorometer at this stage corresponds to the concentration after your sample was diluted into the assay tube. You can record this value and perform the calculation yourself to find out the concentration of your original sample (see *Calculating the Concentration of Your Sample*, below) or the Qubit® 2.0 Fluorometer performs this calculation for you (see *Dilution Calculator*, next page).

1.14 To read the next sample, remove the sample from the Qubit® 2.0 Fluorometer, insert the next sample, and press **Read Next Sample**.

1.15 Repeat sample readings until all samples have been read.

Calculating the Concentration of Your Sample

The Qubit® 2.0 Fluorometer gives values for the Qubit™ dsDNA HS assay in ng/mL. This value corresponds to the concentration after your sample was diluted into the assay tube. To calculate the concentration of your sample, use the following equation:

$$\text{Concentration of your sample} = \text{QF value} \times \left(\frac{200}{x}\right)$$

where:

QF value = the value given by the Qubit® 2.0 Fluorometer

x = the number of microliters of sample you added to the assay tube

This equation generates a result with the same units as the value given by the Qubit® 2.0 Fluorometer (i.e., if the Qubit® 2.0 Fluorometer gave a cncentration in ng/mL, the result of the equation will be in ng/mL).

Appendix F: Protocol TAE-buffer

50X TAE-buffer

- 242 g Tris base
- 57.1 ml Glacial acetic acid
- 100 ml 0.5 EDTA (pH 8.0)
- Add dH_2O until the total volume is 1000 ml

1X TAE-buffer

- 40 ml 50X TAE-buffer
- 1960 ml MQ-water

Appendix G: QIAquick PCR Purification Kit

Quick-StartProtocol

QIAquick® PCR Purification Kit

The QIAquick PCR Purification Kit (cat. nos. 28104 and 28106) can be stored at room temperature (15–25°C) for up to 12 months.

For more information, please refer to the *QIAquick Spin Handbook, March 2008*, which can be found at: www.qiagen.com/handbooks.

For technical assistance, please call toll-free 00800-22-44-6000, or find regional phone numbers at www.qiagen.com/contact.

Notes before starting

- Add ethanol (96–100%) to Buffer PE before use (see bottle label for volume).
- All centrifugation steps are carried out at 17,900 x *g* (13,000 rpm) in a conventional table-top microcentrifuge at room temperature.
- Add 1:250 volume pH indicator I to Buffer PB. The yellow color of Buffer PB with pH indicator I indicates a pH of ≤7.5. If the purified PCR product is to be used in sensitive microarray applications, it may be beneficial to use Buffer PB without the addition of pH indicator I. Do not add pH indicator I to buffer aliquots.

1. Add 5 volumes Buffer PB to 1 volume of the PCR reaction and mix. If the color of the mixture is orange or violet, add 10 µl 3 M sodium acetate, pH 5.0, and mix. The color of the mixture will turn yellow.
2. Place a QIAquick column in ▲ a provided 2 ml collection tube or into ● a vacuum manifold. For details on how to set up a vacuum manifold, refer to the *QIAquick Spin Handbook*.
3. To bind DNA, apply the sample to the QIAquick column and ▲ centrifuge for 30–60 s or ● apply vacuum to the manifold until all the samples have passed through the column. ▲ Discard flow-through and place the QIAquick column back in the same tube.

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4. To wash, add 0.75 ml Buffer PE to the QIAquick column ▲ centrifuge for 30–60 s or ● apply vacuum. ▲ Discard flow-through and place the QIAquick column back in the same tube.
5. Centrifuge the QIAquick column once more in the provided 2 ml collection tube for 1 min to remove residual wash buffer.
6. Place each QIAquick column in a clean 1.5 ml microcentrifuge tube.
7. To elute DNA, add 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or water (pH 7.0–8.5) to the center of the QIAquick membrane and centrifuge the column for 1 min. For increased DNA concentration, add 30 µl elution buffer to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge.
8. If the purified DNA is to be analyzed on a gel, add 1 volume of Loading Dye to 5 volumes of purified DNA. Mix the solution by pipetting up and down before loading the gel.

Appendix H: Sequence alignment pure strains

Pseudoalteromonas

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CLUSTAL 2.1 multiple sequence alignment


R3              -------GGGGGANNGTTT-GATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACAC 52
R6              -------------AGTTTT-GATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACAC 46
U16             ------GGGGGANGANTTT-GATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACAC 53
R1              -GGGCCNGGGGGANAGTTT-GATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACAC 58
R4              GGGGCCNGGGGGANAGTTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACAC 60
U15             ----CCGGGGGNANAGTTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACAC 56
R13             -GGGCCNGGGGGANAGTTT-GATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACAC 58
R14             -GGGCCNGGGGGANAGTTT-GATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACAC 58
R5              -------------ANNTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACAC 46
R7              -GGGCCGGGGGNANAGTTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACAC 59
R12             -GGGCCGGGGGNANAGTTT-GATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACAC 58
                         ** ****************************************

R3              ATGCAAGTCGAGCGGTAACAGAGAGTAGCTTGCTACTTTGCTGACGAGCGGCGGACGGGT 112
R6              ATGCAAGTCGAGCGGTAACAGAGAGTAGCTTGCTACTTTGCTGACGAGCGGCGGACGGGT 106
U16             ATGCAAGTCGAGCGGTAACAGAGAGTAGCTTGCTACTTTGCTGACGAGCGGCGGACGGGT 113
R1              ATGCAAGTCGAGCGGTAACAGAGAGTAGCTTGCTACTTTGCTGACGAGCGGCGGACGGGT 118
R4              ATGCAAGTCGAGCGGTAACAGAGAGTAGCTTGCTACTTTGCTGACGAGCGGCGGACGGGT 120
U15             ATGCAAGTCGAGCGGTAACAGAAAGTAGCTTGCTACTTTGCTGACGAGCGGCGGACGGGT 116
R13             ATGCAAGTCGAGCGGTAACAGAAAGTAGCTTGCTACTTTGCTGACGAGCGGCGGACGGGT 118
R14             ATGCAAGTCGAGCGGTAACATTTC-TAGCTTGCTAGAA-GATGACGAGCGGCGGACGGGT 116
R5              ATGCAAGTCGAGCGGTAACAGAGAGTAGCTTGCTACTTTGCTGACGAGCGGCGGACGGGT 106
R7              ATGCAAGTCGAGCGGTAACAGAGAGTAGCTTGCTACTTTGCTGACGAGCGGCGGACGGGT 119
R12             ATGCAAGTCGAGCGGTAACAGAGAGTAGCTTGCTACTTTGCTGACGAGCGGCGGACGGGT 118
                ********************     **********     * ********************

R3              GAGTAATGCTTGGGAACATGCCTTGAGGTGGGGGACAACAGTTGGAAACGACTGCTAATA 172
R6              GAGTAATGCTTGGGAACATGCCTTGAGGTGGGGGACAACAGTTGGAAACGACTGCTAATA 166
U16             GAGTAATGCTTGGGAACATGCCTTGAGGTGGGGGACAACAGTTGGAAACGACTGCTAATA 173
R1              GAGTAATGCTTGGGAACATGCCTTGAGGTGGGGGACAACAGTTGGAAACGACTGCTAATA 178
R4              GAGTAATGCTTGGGAACATGCCTTGAGGTGGGGGACAACAGTTGGAAACGACTGCTAATA 180
U15             GAGTAATGCTTGGGAACATGCCTTGAGGTGGGGGACAACAGTTGGAAACGACTGCTAATA 176
R13             GAGTAATGCTTGGGAACATGCCTTTAGGTGGGGGACAACAGTTGGAAACGACTGCTAATA 178
R14             GAGTAATGCTTGGGAACATGCCTTTAGGTGGGGGACAACAGTTGGAAACGACTGCTAATA 176
R5              GAGTAATGCTTGGGAATATGCCTTTTGGTGGGGGACAACAGTTGGAAACGACTGCTAATA 166
R7              GAGTAATGCTTGGGAATATGCCTTTTGGTGGGGGACAACAGTTGGAAACGACTGCTAATA 179
R12             GAGTAATGCTTGGGAATATGCCTTTTGGTGGGGGACAACAGTTGGAAACGACTGCTAATA 178
                ***************** *******  ************************************

R3              CCGCATAATGTCTACGGACCAAAGGGGG----CTTCGG--CTCTCGCCTTTAGATTGGCC 226
R6              CCGCATAATGTCTACGGACCAAAGGGGG----CTTCGG--CTCTCGCCTTTAGATTGGCC 220
U16             CCGCATAATGTCTACGGACCAAAGGGGG----CTTCGG--CTCTCGCCTTTAGATTGGCC 227
R1              CCGCATAATGTCTACGGACCAAAGGGGG----CTTCGG--CTCTCGCCTTTAGATTGGCC 232
R4              CCGCATAATGTCTACGGACCAAAGGGGG----CTTCGG--CTCTCGCCTTTAGATTGGCC 234
U15             CCGCATAACGTCTACGGACCAAAGGGGG----CTTCGG--CTCTCGCCTTTAGATTGGCC 230
R13             CCGCATGATGTCTACGGACCAAAGGGGG----CTTCGG--CTCTCGCCTAAAGATTGGCC 232
R14             CCGCATAATGTCTACGGACCAAAGGGGG----CTTCGG--CTCTCGCCTTTAGATTGGCC 230
R5              CCGCATAATGTCTACGGACCAAAGTGGGGGACCTTCGGGCCTCACGCCAAAAGATTAGCC 226
R7              CCGCATAATGTCTACGGACCAAAGTGGGGGACCTTCGGGCCTCACGCCAAAAGATTAGCC 239
R12             CCGCATGATGTCTACGGACCAAAGTGGGGGACCTTCGGGCCTCACGCCAAAAGATTAGCC 238
                ****** * *************** ***    ******  *** ****   ***** ***

R3              CAAGTGGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCCCTAGCTGGT 286
R6              CAAGTGGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCCCTAGCTGGT 280
U16             CAAGTGGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCCCTAGCTGGT 287
R1              CAAGTGGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCCCTAGCTGGT 292
R4              CAAGTGGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCCCTAGCTGGT 294
U15             CAAGTGGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCCCTAGCTGGT 290
R13             CAAGTGGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCAACGATCCCTAGCTGGT 292
R14             CAAGTGGGATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATCCCTAGCTGGT 290
R5              CAAGTGGGATTAGCTAGTTGGTAAGGTAATGGCTTACCAAGGCAACGATCCCTAGCTGGT 286
R7              CAAGTGGGATTAGCTAGTTGGTAAGGTAATGGCTTACCAAGGCAACGATCCCTAGCTGGT 299
R12             CAAGTGGGATTAGCTAGTTGGTAAGGTAATGGCTTACCAAGGCAACGATCCCTAGCTGGT 298
                ************************ ****** **** ******** ****************

R3              TTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAG 346
R6              TTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAG 340
U16             TTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAG 347
R1              TTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAG 352
R4              TTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAG 354
U15             TTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAG 350
R13             TTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAG 352
R14             TTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAG 350
R5              TTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAG 346
R7              TTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAG 359
R12             TTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAG 358
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R3              CAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGA 406
R6              CAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGA 400
U16             CAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGA 407
R1              CAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGA 412
R4              CAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGA 414
U15             CAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGA 410
R13             CAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGA 412
R14             CAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGA 410
R5              CAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGA 406
R7              CAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGA 419
R12             CAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGA 418
                ************************** ********************************

R3              AGGCCTTCGGGTTGTAAAGCACTTTCAGTCAGGAGGAAAGGTTAGTAGTTAATACCTGCT 466
R6              AGGCCTTCGGGTTGTAAAGCACTTTCAGTCAGGAGGAAAGGTTAGTAGTTAATACCTGCT 460
U16             AGGCCTTCGGGTTGTAAAGCACTTTCAGTCAGGAGGAAAGGTTAGTAGTTAATACCTGCT 467
R1              AGGCCTTCGGGTTGTAAAGCACTTTCAGTCAGGAGGAAAGGTTAANNGTTAATACCTGCT 472
R4              AGGCCTTCGGGTTGTAAAGCACTTTCAGTCAGGAGGAAAGGTTAGTAGTTAATACCTGCT 474
U15             AGGCCTTCGGGTTGTAAAGCACTTTCAGTCAGGAGGAAAGGTTAGTAGTTAATACCTGCT 470
R13             AGGCCTTCGGGTTGTAAAGCACTTTCAGTAAGGAGGAAAGGTTAGTAGTTAATACCTGCT 472
R14             AGGCCTTCGGGTTGTAAAGCACTTTCAGTAAGGAGGAAAGGTTAGTAGTTAATACCTGCT 470
R5              AGGCCTTCGGGTTGTAAAGCACTTTCAGTAAGGAGGAAAGGGTAGTCTTTAATAGAGGCT 466
R7              AGGCCTTCGGGTTGTAAAGCACTTTCAGTAAGGAGGAAAGGGTAGTCTTTAATAGAGGCT 479
R12             AGGCCTTCGGGTTGTAAAGCACTTTCAGTCAGGAGGAAAGGGTGTGTTTTAATAGAGCAT 478
                ****************************** *********** *      ******     *

R3              AGCTGTGACGTTACTGACAGAAGAAGCACCGGCTAACTCCGTGCCAGCA-CNGGCGGTAA 525
R6              AGCTGTGACGTTACTGACAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGNCGGTAA 520
U16             AGCTGTGACGTTACTGACAGAAGAAGCACCGGCTAACTCCGTGCCAGCA-CCGNCGGTAA 526
R1              AGCTGTGACGTTACTGACAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGNNGGTAA 532
R4              AGCTGTGACGTTACTGACAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGNNGGTAA 534
U15             AGCTGTGACGTTACTGACAGAAGAAGCACCGGCTAACTCCGTGCCAGCACC--------- 521
R13             AGCTGTGACGTTACTTACAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGNAGGTAA 532
R14             AGCTGTGACGTTACTTACAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGNCGGTAA 530
R5              ATCTGTGACGTTACTTACAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCG------- 519
R7              ATCTGTGACGTTACTTACAGAAGAAGCACCGGCTAACTCCGTGCCANCANC--------- 530
R12             ATCTGTGACGTTACTGACAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGNNGGTAA 538
                * ************** ******************************** ** *
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Vibrio

CLUSTAL 2.1 multiple sequence alignment

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R2              GGGCCNGGGGGANAGTTT-GATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACA 59
R10             GGGCCNGGGGGANAGTTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACA 60
R8              --GCCNGGGGGANAGTTT-GATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACA 57
R9              GGGCCNGGGGGANAGTTT-GATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACA 59
                ***************** ****************************************

R2              TGCAAGTCGAGCGGAAACGACAACATTGAATCTTCGGAGGATTTGTTGGGCGTCGAGCGG 119
R10             TGCAAGTCGAGCGGAAACGACANNATTGAANCTTCGGAGGATTTNNTGGGCGTCGAGCGG 120
R8              TGCAAGTCGAGCGGAAACGACACTAACAATCCTTCGGNTGCGTTAATGGGCGTCGAGCGG 117
R9              TGCAAGTCGAGCGGAAACGACACTAACAATCCTTCGGGTGCGTTAATGGGCGTCGAGCGG 119
                ************************  *   *  *******  *  **  ***************


R2              CGGACGGGTGAGTAATGCCTAGGAAATTGCCTTGATGTGGGGGATAACCATTGGAAACGA 179
R10             CGGACGGGTGAGTAATGCCTAGGAAATTGCCTTGATGTGGGGGATAACCATTGGAAACGA 180
R8              CGGACGGGTGAGTAATGCCTAGGAAATTGCCTTGATGTGGGGGATAACCATTGGAAACGA 177
R9              CGGACGGGTGAGTAATGCCTAGGAAATTGCCTTGATGTGGGGGATAACCATTGGAAACGA 179
                ************************************************************

R2              TGGCTAATACCGCATAATGCCTACGGGCCAAAGAGGGGGATCTTCGGACCTCTCGCGTCA 239
R10             TGGCTAATACCGCATAATGCCTACGGGCCAAAGAGGGGGANCTTCGGACCTCTCGCGTCA 240
R8              TGGCTAATACCGCATAATGCCTACGGGCCAAAGAGGGGGATCTTCGGACCTCTCGCGTCA 237
R9              TGGCTAATACCGCATAATGCCTACGGGCCAAAGAGGGGGATCTTCGGACCTCTCGCGTCA 239
                ************************************************ ************

R2              AGATATGCCTAGGTGGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCC 299
R10             AGATATGCCTAGGTGGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCC 300
R8              AGATATGCCTAGGTGGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCC 297
R9              AGATATGCCTAGGTGGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCC 299
                ************************************************************

R2              CTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTAC 359
R10             CTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTAC 360
R8              CTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTAC 357
R9              CTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTAC 359
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R2              GGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCCATGCCGCGT 419
R10             GGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCCATGCCGCGT 420
R8              GGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCCATGCCGCGT 417
R9              GGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCCATGCCGCGT 419
                ************************************************************

R2              GTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGTTGTGAGGAAGGGGGTAACGTTA 479
R10             GTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGTTGTGAGGAAGGGGGTAACGTTA 480
R8              GTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGTTGTGAGGAAGGGGGTAACGTTA 477
R9              GTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGTTGTGAGGAAGGGNGTNNNGTTA 479
                ************************************************** **    ****

R2              ATAGCGTTATCTCTTGACGTTAGCAACAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGC 539
R10             ATAGCGTTATCTCTTGACGTTAGCAACAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGC 540
R8              ATAGCGTTATCTCTTGACGTTAGCAACAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGC 537
R9              ATAGCGTNNTCTCTTGACGTTAGCAACAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGC 539
                *******  ************************************************

R2              CGNNGGTAA----- 548
R10             CGCANGTAANAAA- 553
R8              CGACGGTAANAAAA 551
R9              CGNNGGTAA----- 548
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Thalassobacter

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CLUSTAL 2.1 multiple sequence alignment

K19             ---------AGTTTGATCCTGGCTCAGAACGAACGCTGGCGGCAGGCCTAACACATGCAA 51
K20             CNGGGGGANAGTTTGATCCTGGCTCAGAACGAACGCTGGCGGCAGGCCTAACACATGCAA 60
                          **************************************************

K19             GTCGAGCGCCACCTTCGGGTGGAGCGGCGGACGGGTTAGTAACGCGTGGGAATATGCCCT 111
K20             GTCGAGCGCCACCTTCGGGTGGAGCGGCGGACGGGTTAGTAACGCGTGGGAATATGCCCT 120
                ************************************************************

K19             TTACTAAGGAATAGCCACTGGAAACGGTGAGTAATACCTTATACGCCCTTCGGGGGAAAG 171
K20             TTACTAAGGAATAGCCACTGGAAACGGTGAGTAATACCTTATACGCCCTTCGGGGGAAAG 180
                ************************************************************

K19             ATTTATCGGTAAAGGATTAGCCCGCGTAAGATTAGATAGTTGGTGGGGTAACGGCCTACC 231
K20             ATTTATCGGTAAAGGATTAGCCCGCGTAAGATTAGATAGTTGGTGGGGTAACGGCCTACC 240
                ************************************************************

K19             AAGTCGACGATCTTTAGCTGGTTTGAGAGGATGATCAGCAACACTGGGACTGAGACACGG 291
K20             AAGTCGACGATCTTTAGCTGGTTTGAGAGGATGATCAGCAACACTGGGACTGAGACACGG 300
                ************************************************************

K19             CCCAGACTCCTACGGGAGGCAGCAGTGGGGAATCTTAGACAATGGGCGCAAGCCTGATCT 351
K20             CCCAGACTCCTACGGGAGGCAGCAGTGGGGAATCTTAGACAATGGGCGCAAGCCTGATCT 360
                ************************************************************

K19             AGCCATGCCGCGTGAGTGATGAAGGCCCTAGGGTCGTAAAGCTCTTTCGCCAGAGATGAT 411
K20             AGCCATGCCGCGTGAGTGATGAAGGCCCTAGGGTCGTAAAGCTCTTTCGCCAGAGATGAT 420
                ************************************************************

K19             AATGACAGTATCTGGTAAAGAAACCCCGGCTAACTCCGTGCCAGCAGCCGACGGTAANAA 471
K20             AATGACAGTATCTGGTAAAGAAACCCCGGCTAACTCCGTGCCAGCA-CCGGCGGTAANAA 479
                ********************************************** *** **********
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Aquimarina

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CLUSTAL 2.1 multiple sequence alignment


K18             GCGGCGGGCGGGCGGGGCCGGGGGGAGAGTTTGATCCTGGCTCAGGATGAACGCTAGCGG 60
K21             -------------------------------------------GATGAACGCTAGCGG 15
                                                           ***************

K18             CAGGCTTAACACATGCAAGTCGAGGGGCAGCATTGGTA-CTTG-TATCAGATGGCGACCG 117
K21             CAGGCTTAACACATGCAAGTCGAGGGGTAACA--GGTAGCTTGCTAT--GCTGACGACCG 71
                **************************** *  **   **** **** ***   * **  ******

K18             GCGCACGGGTGCGTAACGCGTATAGAACCTACCTTATATTAAGGGATAGCCCAGAGAAAT 177
K21             GCGCACGGGTGCGTAACGCGTATAGAACCTACCTTATAGTAAGGGATAGCCCAGAGAAAT 131
                ************************************ ********************

K18             TTGGATTAATACCTTATAGTATCAGTAAGCAGCATTGCTTTATGATTAAAGATTCATCGA 237
K21             TTGGATTAATACCTTATGGTATTGGACTGTGGCATCGCAGACTAATTAAAGATTTATTGC 191
                ***************** ****  *   *  **** **     * ********** ** *

K18             TATAAGATGGCTATGCGTTCTATTAGCTAGTTGGTATGGTAACGGCATACCAAGGCTACG 297
K21             TATAAGATGGCTATGCGTTCTATTAGCTAGTTGGTATGGTAACGGCATACCAAGGCTACG 251
                ************************************************************

K18             ATAGATAGGGGTCCTGAGAGGGAGATCCCCCACACTGGTACTGAGACACGGACCAGACTC 357
K21             ATAGATAGGGGTCCTGAGAGGGAGATCCCCCACACTGGTACTGAGACACGGACCAGACTC 311
                ************************************************************

K18             CTACGGGAGGCAGCAGTGAGGAATATTGGACAATGGAGGCAACTCTGATCCAGCCATGCC 417
K21             CTACGGGAGGCAGCAGTGAGGAATATTGGACAATGGAGGCAACTCTGATCCAGCCATGCC 371
                ************************************************************

K18             GCGTGTAGGAAGACTGCCCTATGGGTTGTAAACTACTTTTATAGAGGAAGAAACGCAGAT 477
K21             GCGTGTAGGAAGACTGCCCTATGGGTTGTAAACTACTTTTATAGAGGAAGAAACGACATT 431
                ************************************************************

K18             ACGTGTATTTGTTTGACGGTACTCTACGAATAAGGATCGCCTAACTCCGTGCCACAGCCT 537
K21             ACGTGTAATGTTTTGACGGTACTCTACGAATAAGGATCGGCTAACTCCGTG--------- 482
                ******* *   ***************************** ***********

K18             AAATAATAAGGGGCTGGGCAGGAGCGGCGGAAACAGGAGAGCATCGTAATAAATATTTGA 597
K21             ------------------------------------------------------------
```

Pacificibacter

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CLUSTAL 2.1 multiple sequence alignment


K23             AACGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGCTTCCCTTCGGGGAGGAGC 60
K24             AACGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGCTTCCCTTCGGGGAGGAGC 60
                ************************************************************

K23             GGCGGACGGGTTAGTAACGCGTGGGAATATACCCTTCACTATGGAATAGCCTCGGGAAAC 120
K24             GGCGGACGGGTTAGTAACGCGTGGGAATATACCCTTCACTATGGAATAGCCTTTGGAAAC 120
                ************************************************   ******

K23             TGAGAGTAATACCATATACGCCCTTCGGGGGAAAGATTTATCGGTGAGGGATTAGCCCGC 180
K24             GAAGAGTAATACCATATACGCCCTTCGGGGGAAAGATTTATCGGTGAGGGATTAGCCCGC 180
                  ************************************************************

K23             GTAAGATTAGATAGTTGGTGGGGTAATGGCCTACCAAGTCTACGATCTTTAGCTGGTTTG 240
K24             GTAAGATTAGATAGTTGGTGGGGTAATGGCCTACCAAGTCTACGATCTTTAGCTGGTTTG 240
                ************************************************************

K23             AGAGGATGATCAGCAACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAG 300
K24             AGAGGATGATCAGCAACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAG 300
                ************************************************************

K23             TGGGGAATCTTAGACAATGGGCGCAAGCCTGATCTAGCCATGCCGCGTGAGTGATGAAGG 360
K24             TGGGGAATCTTAGACAATGGGCGCAAGCCTGATCTAGCCATGCCGCGTGAGTGATGAAGG 360
                ************************************************************

K23             CCTTAGGGTCGTAAAGCTCTTTCACCTGTGAAGATAATGACGGTAGCAGGAAAAGAAACC 420
K24             CCTTAGGGTCGTAAAGCTCTTTCACCTGTGAAGATAATGACGGTAGCAGGAAAAGAAACC 420
                ************************************************************

K23             CCGGCTAACTCCGTGCCACANC 442
K24             CCGGCTAACTCCGTG------- 435
```

Appendix I: SYBR Green II - RNA complex stability

Table I.1: SYBR®Green II intensity of samples exposed to light and covered with aluminium foil (dark) prior to analysis. The percentage reduction was calculated from the first sample exposed to light. All samples were incubated in the dark for 15 minutes prior to analysis (not included in the time.

Sample	Incubation time (Min)	Mean Intensity FL1 Light	Mean Intensity FL1 Dark	Percentage reduction in intensity
1	3.46	78 743		0.00
2	6.92		71 934	8.65
3	10.38	77 040		2.16
4	13.83		74 421	5.49
5	17.29	73 133		7.12
6	20.75		72 245	8.25
7	24.21	68 676		12.78
8	27.67		71 018	9.81
9	31.13	68 933		12.46
10	34.58		70 002	11.10
11	38.04	70 133		10.93
12	41.50		69 436	11.82
13	44.96	67 524		14.25
14	48.42		68 077	13.55
15	51.88	64 347		18.28
16	55.33		68 144	13.46
17	58.79	67 271		14.57
18	62.25		68 069	13.56
19	65.71	67 900		13.77
20	69.17		66 574	15.45
21	72.63	63 812		18.96
22	76.08		66 059	16.11
23	79.54	65 139		17.28
24	83.00		67 155	14.72

Appendix J: Maximum/minimum RNA content of pure strains

Table J.1: Results from flow cytometry analysis of the pure cultures. Maximum RNA content (mean FL1 4h or 8h) and minimum RNA content (mean FL1 S) is given. Numbers in red are the numbers used for calculation of maximum FL1 intensity in the ratio max/min intensity.

ID	1/T	Mean FL1 4h	Mean FL1 8h	Mean FL1 S	Ratio FL1
R1	0.0208	258 025	227 889	40 463	6.38
R2	0.0208	273 639	235 504	46 151	5.93
R3	0.0208	161 854	227 069	41 612	5.46
R4	0.0208	169 035	200 658	42 492	4.72
R5	0.0208	215 084	223 044	49 719	4.49
R6	0.0208	162 282	221 555	41 645	5.32
R7	0.0208	193 149	206 422	40 540	5.09
R8	0.0208	246 821	320 928	35 913	8.94
R9	0.0208	236 421	248 078	33 524	7.40
R10	0.0208	226 437	286 149	37 146	7.70
R11	0.0208	139 610	210 237	53 573	3.92
R12	0.0139	146 607	195 295	51 176	3.82
R13	0.0139	163 202	188 823	49 246	3.83
R14	0.0139	183 251	192 249	37 748	5.09
U15	0.0104	105 501	154 206	56 856	2.71
U16	0.0104	206 298	196 041	35 525	5.81
K17	0.0083	159 771	155 275	93 812	1.70
K18	0.0083	75 518	75 643	7 884	9.59
K19	0.0069	50 418	57 455	52 052	1.10
K20	0.0060	61 069	65 886	21 230	3.10
K21	0.0060	84 675	91 074	24 792	3.67
K23	0.0038	50 672	53 425	32 539	1.64
K24	0.0035	23634	24 377	30 862	0.79

Appendix K: RNA content and cell densities of surface and 90 meter seawater communities

Table K.1: Flow cytometry analysis results for seawater samples (surface and 90 meters depth), with RNA content per cell (mean FL1) and cells per µl for the unpulsed sample (0 hour) and for the subsamples incubated at 6 hours with nutrients added. The ratios between the RNA content and cell counts at 0 and 6 hours, in addition to the number of cell doublings are also shown.

Surface water	Mean FL1 0 hour	Mean FL1 6 hours	Ratio FL1 6h/0h	Cells/ µL 0 hours	Cells / µL 6 hours	Ratio events 6h/0h	Cell doublings
Day 0	23 269	35 702	1.53	526	624	1.19	0.25
1	51 836	50 292	0.97	24 040	58 850	2.45	1.29
2	36 078	52 545	1.46	39 510	60 310	1.53	0.61
3	45 493	64 330	1.41	37 160	51 570	1.39	0.47
4	30 868	56 423	1.83	2 060	690	0.33	-1.58
5	26 884	51 717	1.92	1 770	780	0.44	-1.18
7	26 012	31 903	1.23	3 230	2 820	0.87	-0.20
9	38 325	65 180	1.70	1 400	1 120	0.80	-0.32
13	29 512	49 730	1.69	1 230	1 260	1.02	0.03
17	32 106	43 538	1.36	1 230	1 150	0.93	-0.10
20	28 387	38 965	1.37	860	890	1.03	0.05
24	27 129	38 134	1.41	720	640	0.89	-0.17
90 m. water	**Mean FL1 0 hour**	**Mean FL1 6 hours**	**Ratio FL1 6h/0h**	**Cells / µL 0 hour**	**Cells / µL 6 hours**	**Ratio events 6h/0h**	**Cell doublings**
Day 0	26 187	28 051	1.07	128	140	1.09	0.13
1	83 990	63 761	0.76	1 844	5 714	3.10	1.63
2	37 638	55 580	1.48	8 300	12 858	1.55	0.63
3	44 521	54 792	1.23	38 260	55 740	1.46	0.54
4	36 020	52 629	1.46	40 500	57 760	1.43	0.51
5	33 046	53 544	1.62	39 950	55 480	1.39	0.47
7	33 150	59 752	1.80	26 810	34 120	1.27	0.35
9	26 619	47 505	1.78	2 650	1 720	0.65	-0.62
13	24 105	37 878	1.57	2 070	2 100	1.01	0.02
17	24 782	33 708	1.36	2 140	1 570	0.73	-0.45
20	26 583	40 841	1.54	860	740	0.86	-0.22
24	27 705	37 122	1.34	770	540	0.70	-0.51

Appendix L: Fluorescent subdivisions with percentage cells

Table L.1: Range for the 12 the fluorescent subdivisions is given as low bound FL1 intensity and high bound FL1 intensity. Results showed for surface (A) and 90 meter seawater (B) with percentage cells within each subdivisions are given for 0-5, 7, 9, 13, 17, 20 and 24 days.

Low bound	High bound	A0	A1	A2	A3	A4	A5	A7	A9	A13	A17	A20	A24
10 000	14 000	13.04	3.44	3.30	2.63	22.48	27.78	21.44	9.00	10.14	10.08	12.70	14.69
14 000	19 600	13.14	4.73	4.94	4.93	14.01	15.21	24.80	8.14	11.54	13.68	17.49	17.86
19 600	27 440	17.11	7.28	7.57	7.48	8.93	7.57	18.58	7.95	11.78	15.96	19.00	18.82
27 440	38 416	19.49	21.38	16.95	9.25	6.74	3.75	11.23	8.25	15.97	20.04	18.34	16.05
38 416	53 782	16.17	30.75	30.72	20.90	5.76	3.64	3.54	12.99	17.65	13.95	8.87	9.80
53 782	75 295	9.37	19.55	20.70	25.04	5.57	3.91	1.89	16.25	10.07	8.45	8.28	6.23
75 295	105 414	4.19	8.07	10.73	18.77	6.99	6.59	3.20	19.36	10.65	8.22	7.06	4.96
105 414	147 579	1.53	2.31	2.60	7.30	9.20	8.20	3.85	9.53	5.74	4.30	2.95	3.66
147 579	206 611	0.78	1.05	1.06	1.79	6.19	5.28	1.78	3.25	1.52	1.12	0.97	1.14
206 611	289 255	0.28	0.47	0.48	0.75	2.63	2.24	0.53	1.29	0.55	0.59	0.44	0.60
289 255	404 957	0.05	0.21	0.22	0.37	0.86	1.02	0.24	0.53	0.54	0.31	0.52	0.40
404 957	566 939	0.03	0.08	0.09	0.17	0.21	0.38	0.12	0.33	0.24	0.19	0.06	0.22
Low bound	**High bound**	**B0**	**B1**	**B2**	**B3**	**B4**	**B5**	**B7**	**B9**	**B13**	**B17**	**B20**	**B24**
10 000	14 000	26.32	3.51	1.18	1.25	1.51	1.77	3.06	9.58	18.15	20.2	21.84	24.1
14 000	19 600	21.63	3.56	1.88	2.54	3.16	3.24	6.55	11.6	18.68	20.33	22.29	22.95
19 600	27 440	14.38	5.3	4.49	5.5	6.64	6.75	13.31	27.39	24.01	25.49	22.02	20.46
27 440	38 416	12.61	9.36	17.42	15.6	17.8	15.87	12.47	20.64	17.8	15.44	13.29	12.45
38 416	53 782	9.95	23.3	35.26	36.7	35.1	33.6	18.42	12.83	8.71	8.23	6.17	5.68
53 782	75 295	6.18	28.3	23.88	22.2	22.2	24.31	20.99	3.9	2.68	1.91	1.98	1.93
75 295	105 414	2.37	17.7	11.13	12.2	9.85	10.44	15.35	2.67	1.21	0.94	1.26	1.34
105 414	147 579	1.13	4.75	2.76	2.42	2.21	2.37	5.8	2.64	1.24	0.84	1.07	1.26
147 579	206 611	0.19	2.12	1.11	0.88	0.8	0.83	2.14	3.45	1.43	1.07	1.67	1.85
206 611	289 255	0.23	0.95	0.48	0.37	0.36	0.36	0.97	2.44	1.81	1.58	2.43	2.84
289 255	404 957	0.04	0.43	0.18	0.18	0.17	0.17	0.43	1.07	1.23	1.00	2.06	1.26
404 957	566 939	0.1	0.16	0.07	0.07	0.07	0.08	0.2	0.29	0.39	0.24	0.62	0.13

Table M.1: Subdivisions added together for the surface (A) and the 90 meter (B) seawater in samples from 0-5, 7, 9, 13, 17, 20 and 24 days. Percentage cells of K- and r-strategists within each day are given for the different subdivisions included. The range is given as high bound and low bound of fluorescent intensity.

Percentage K-strategists																	
#Subdivisions	**Low bound**	**High bound**	**A0**	**A1**	**A2**	**A3**	**A4**	**A5**	**A7**	**A9**	**A13**	**A17**	**A20**	**A24**	**Average**	**SD**	**CV**
2	10 000	19 600	26.18	8.17	8.24	7.56	36.49	42.99	46.2	17.1	21.68	23.76	30.19	32.55	25.1	13.3	0.53
3	10 000	27 440	43.29	15.45	15.81	15.04	45.42	50.56	64.82	25.09	33.46	39.72	49.19	51.37	37.4	16.5	0.44
4	10 000	38 416	62.78	36.83	32.76	24.29	52.16	54.31	76.05	33.34	49.43	59.76	67.53	67.42	51.4	16.4	0.32
5	10 000	53 782	78.95	62.78	63.48	45.19	57.92	57.95	79.59	46.33	67.08	73.71	76.4	77.22	66.0	12.1	0.18
Percentage r-strategists																	
	Low bound	**High bound**	**A0**	**A1**	**A2**	**A3**	**A4**	**A5**	**A7**	**A9**	**A13**	**A17**	**A20**	**A24**	**Average**	**SD**	**CV**
3	206 611	566 939	0.36	0.76	0.79	1.29	3.70	3.64	0.89	2.15	1.33	1.09	1.02	1.22	1.5	1.1	0.72
4	147 579	566 939	0.78	1.05	1.06	1.79	6.19	5.28	1.78	3.25	1.52	1.12	0.97	1.14	2.2	1.8	0.83
5	105 414	566 939	2.31	3.36	3.66	9.09	15.39	13.48	5.63	12.78	7.26	5.42	3.92	4.80	7.3	4.4	0.61
6	75 295	566 939	6.50	11.43	14.39	27.86	22.38	20.07	8.83	32.14	17.91	13.64	10.98	9.76	16.3	8.0	0.49
7	53 782	566 939	15.87	30.98	35.09	52.90	27.95	23.98	10.72	48.39	27.98	22.09	19.26	15.99	27.6	12.8	0.46
8	38 416	566 939	32.04	61.73	65.81	73.80	33.71	27.62	14.26	61.38	45.63	36.04	28.13	25.79	42.2	19.0	0.45
Percentage K-strategists																	
	Low bound	**High bound**	**B0**	**B1**	**B2**	**B3**	**B4**	**B5**	**B7**	**B9**	**B13**	**B17**	**B20**	**B24**	**Average**	**SD**	**CV**
2	10 000	19 600	47.95	7.07	3.06	3.79	4.67	5.01	9.61	21.18	36.83	40.53	44.13	47.05	22.6	19.1	0.85
3	10 000	27 440	62.33	12.37	7.55	9.29	11.31	11.76	22.92	48.57	60.84	66.02	66.15	67.51	37.2	26.5	0.71
4	10 000	38 416	74.94	21.73	24.97	24.88	29.12	27.63	35.39	69.21	78.64	81.46	79.44	79.96	52.3	26.5	0.51
5	10 000	53 782	84.89	45.02	60.23	61.56	64.17	61.23	53.81	82.04	87.35	89.69	85.61	85.64	71.8	15.6	0.22
Percentage r-strategists																	
	Low bound	**High bound**	**B0**	**B1**	**B2**	**B3**	**B4**	**B5**	**B7**	**B9**	**B13**	**B17**	**B20**	**B24**	**Average**	**SD**	**CV**
3	206 611	566 939	0.37	1.54	0.73	0.62	0.60	0.61	1.60	3.80	3.43	2.82	5.11	4.23	2.12	1.68	0.79
4	147 579	566 939	0.56	3.66	1.84	1.50	1.40	1.44	3.74	7.25	4.86	3.89	6.78	6.08	3.58	2.29	0.64
5	105 414	566 939	1.69	8.41	4.60	3.92	3.61	3.81	9.54	9.89	6.10	4.73	7.85	7.34	5.96	2.62	0.44
6	75 295	566 939	4.06	26.12	15.73	16.09	13.46	14.25	24.89	12.56	7.31	5.67	9.11	8.68	13.2	6.95	0.53
7	53 782	566 939	10.24	54.42	39.61	38.25	35.65	38.56	45.88	16.46	9.99	5.67	11.09	10.61	26.5	17.0	0.64
8	38 416	566 939	20.19	77.71	74.87	74.93	70.7	72.16	64.3	29.29	18.7	15.81	17.26	16.29	26.0	28.0	0.62

Appendix N: Melting curves for *H.neptunium* and *B.licheniformis*

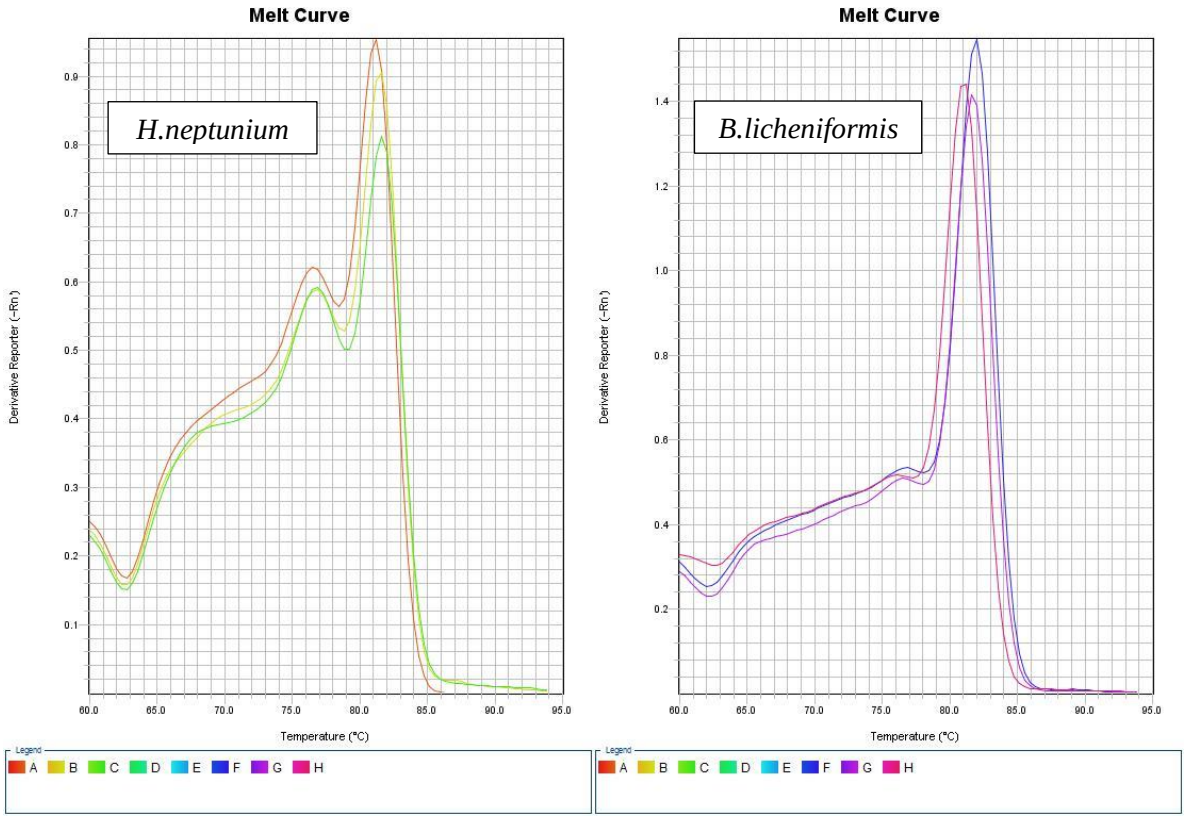

Figure N.1: The melting curves for rtPCR amplicons obtained for *H.neptunium and B.licheniformis*. Derivative reporter (-Rn) is the change in fluorescent divided by change in temperature and is plotted against temperature.