Development of flow cytometry-based techniques for assessing microbial inactivation after water disinfection and downstream processes

Mathilde Lindivat

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Name: Mathilde Lindivat

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Scientific environment

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Abstract

Monitoring aquatic microbial vitality is challenging. Several methods are available, and flow cytometry analysis (FCM) and vitality stains represent an advantageous way to obtain information on microbes. When combining vitality stains with FCM, single cells can be classified into live cells with activity, damaged cells, or dead cells. Applications of FCM to microbial water quality monitoring in ballast water transport and aquaculture may solve limitations from current methods like PCR and cultivation.

Ballast water transport can cause the spread of invasive species in different geographical areas. International regulations require water treatment prior to release to ensure microbial inactivation and/or removal. Well-boats used in the aquaculture industry represent a similar problem by potentially spreading diseases between farms. The efficiency of treatments and effect on cells of various water treatments need documentation and monitoring. In this thesis, two double staining methods combined with FCM were applied to understand and evaluate phytoplankton and bacterial vitality before and after UV and heat treatments.

Firstly, the combination of esterase substrate CFDA-AM and membrane permeability stain SYTOX Blue was used to follow *Tetraselmis suecica* vitality for ballast water UV-based treatment system (BWTS) (**Paper I**). Recovery and regrowth of *T. suecica* cells after treatment with UV doses between 100 and 400 mJ/cm^2 was evaluated after incubation in light and dark. Esterase activity and membrane permeability were affected by high UV doses of 300 and 400 mJ/cm^2. Subsequently, incubation in the light increased the cell damages compared to incubation in the dark. The double staining was efficient to detect live, damaged, and dying cells in all samples. The method is effective to determine vitality of phytoplankton for BWTS.

Secondly, Bioorthogonal non-canonical amino acid tagging (BONCAT) was used to monitor protein synthesis in bacteria (**Paper II**). The procedure consists in an incubation of bacteria with an analogous amino acid followed by a click reaction. The click method links a fluorescent molecule to the analogous amino acid, thus making newly synthesized proteins detectable. The method was adapted to FCM by calibrating

the analogous amino acid concentration, testing of different click dyes, and combination with DNA stains using *Escherichia coli* as a test organism. Implementation of flow cytometry for analysis decreased analysis time compared to microscopy. Application to natural prokaryote communities revealed that only a small population of the sampled organisms were active cells.

Lastly, the determination of bacterial vitality after UV and heat treatment with BONCAT/SYBR Green staining and FCM analysis was explored in **Paper III**. *E. coli* and natural bacterial communities were treated with UV doses between 25 and 200 mJ/cm^2. In addition, heat treatments (from 15 to 45 min at 55 ºC) was tested to observe effects on protein synthesis. Other methods such as plate counts and membrane permeabilization stains Propidium Iodine (PI) and SYTO9 were used to compare vitality levels with BONCAT/SYBR Green staining. Both treatments affected protein synthesis and DNA integrity for monoculture and natural bacterial communities. Protein synthesis activity still remained at 24h (for *E. coli*) and 48h (for natural communities) after treatments.

CFDA-AM/SYTOX Blue and BONCAT/SYBR Green are specific vitality stains covering important cell parameters directly linked to cell vitality. Both double staining methods for phytoplankton and bacteria have proven to be compatible for FCM analysis. Application of FCM to water microbial quality analysis can be considered for aquaculture industry and ballast water monitoring.

Sammendrag

Overvåking av mikrobiell vitalitet i akvatiske miljøer kan være utfordrende. Flere metoder er tilgjengelig, og Flow cytometri (FCM) analyser er en effektiv måte å få informasjon om mikrobene. Å kombinere ulike liv/død-farginger med FCM analyser gjør det mulig å dele inn i populasjoner av levende celler med aktivitet, skadede celler, eller døde celler. Anvendelse av FCM til overvåking av mikrobiell vannkvalitet innen ballastvann transport og akvakultur, kan løse noen av begrensningene i eksisterende metoder som PCR og kultivering.

Transport av ballastvann kan forårsake spredning av invaderende arter i ulike geografiske områder. Internasjonale regelverk krever at ballastvann skal renses før utslipp for å sørge for inaktivering og/eller fjerning av mikrober. Tilsvarende problemer finnes også i akvakulturnæringen, hvor brønnbåter utgjør en risiko for spredning av sykdommer mellom anlegg. Effektivitet av behandling og effekt på celler i de ulike vannbehandlingene krever både dokumentasjon og overvåking. Denne avhandlingen beskriver og diskuterer dobbeltfarging kombinert med FCM som metodikk for å evaluere vitalitet hos fytoplankton og bakterier før og etter UV- og varmebehandling.

Den første dobbeltfargingen jeg undersøkte kombinerer CFDA-AM, som utrykker esterase aktivitet, med SYTOX Blue for membranpermeabilitet til å undersøke vitalitet hos *Tetraselmis suecica* (**Publikasjon I**). Reparasjon og gjenvekst av *T. suecica* celler etter behandling med UV doser mellom 100 og 400 mJ/cm^2 ble evaluert etter inkubasjon i lys og mørke. Både esterase aktivitet og membranpermeabilitet ble påvirket av høye UV doser på 300 og 400 mJ/cm^2, og økningen i celleskader ved inkubasjon i lys var høyere enn ved mørkeinkubasjon. Dobbeltfargingen skilte effektivt mellom levende, skadede og døde celler i alle prøvene. Metoden er egnet til å bestemme vitalitet hos fytoplankton etter behandling i UV-baserte ballastvann behandlingssystemer.

Den andre dobbeltfargingen består av Bioorthogonal non-canonical aminosyre tagging (BONCAT), som gjør det mulig å følge proteinsyntesen hos bakterier, sammen med DNA-farging (**Publikasjon II**). I BONCAT-metodikken blir celler inkubert med

en analog aminosyre, før en klikk-reaksjon med et fluoriserende molekyl gjør det mulig å identifisere cellene med aktiv proteinsyntese. Metoden ble tilpasset til FCM ved å justere konsentrasjonen av den analoge aminosyren, teste ulike klikk-fargestoffer, og kombinere med ulike DNA fargestoffer i *Escherichia coli*. Bruken av FCM reduserte analysetiden sammenlignet med mikroskopi. Anvendelse av metoden for naturlige prokaryote samfunn viste at kun en liten populasjon av cellene i prøvene var aktive.

Publikasjon III beskriver undersøkelser av bakteriell vitalitet ved å kombinere BONCAT/SYBR Green farging med FCM analyser av UV- og varmebehandlede vannprøver. *E. coli* og naturlige bakteriesamfunn ble behandlet med UV-doser mellom 25 og 200 mJ/cm^2. I tillegg ble varmebehandling (fra 15 til 45 min ved 55 ºC) brukt for å observere effekter på proteinsyntesen. Kimtall, samt farging med Propidium Iodine (PI) og SYTO9 etterfulgt av FCM analyser, ble brukt for å sammenlikne resultatene fra BONCAT vitalitets-analysene. Både UV- og varmebehandlingene påvirket proteinsyntesen og DNA integriteten i cellene for både monokultur og naturlige bakteriesamfunn. Proteinsyntesen var fremdeles aktiv 24 og 48 timer etter behandling for hhv *E. coli* og naturlige bakteriesamfunn.

CFDA-AM/SYTOX Blue og BONCAT/SYBR Green er spesifikke fargemetoder som avdekker viktige cellefunksjoner som er direkte koblet til celle vitalitet. De to dobbeltfargingene for fytoplankton og bakterier har vist seg å være kompatible med FCM analyser. Bruk av FCM i vannkvalitetsanalyser bør vurderes for både akvakulturnæringen og ballastvann overvåking.

List of Publications

Paper I

Olsen, R. O., Lindivat, M., Larsen, A., Thuestad, G., & Hoell, I. A. (2019). Incubation in light versus dark affects the vitality of UV-irradiated Tetraselmis suecica differently: A flow cytometric study. *Marine Pollution Bulletin*, *149*, 110528.

Paper II

Lindivat, M., Larsen, A., Hess-Erga, O. K., Bratbak, G., & Hoell, I. A. (2020). Bioorthogonal non-canonical amino acid tagging combined with flow cytometry for determination of activity in aquatic microorganisms. *Frontiers in microbiology*, *11*, 1929.

Paper III

Lindivat, M., Bratbak, G., Larsen, A., Hess-Erga, O. K., & Hoell, I. A. (2021). Flow Cytometric Analysis of Bacterial Protein Synthesis: Monitoring Vitality After Water Treatment. *Frontiers in microbiology*, 3771.

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List of abbreviations

AF647 - AlexaFluor® 467

AHA - L-Azidohomoalanine

ATP - Adenosine Triphosphate

BONCAT - BioOrthogonal Non-Canonical Amino Acid Tagging

cFDA - Carboxyfluorescein Diacetate

CFDA-AM - 5-Carboxyfluorescein Diacetate Acetoxymethyl Ester

CFU - Colony Forming Unit

DNA - Deoxyribonucleic Acid

DOC - Dissolved Organic Carbon

DVC - Direct Viable Count

EMA - Ethidium monoazide

FISH - Fluorescence *in situ* Hybridization

FCM - Flow Cytometry

FSC - Forward Scatter

HNA - High DNA content

HPG - L-Homopropargylglycine

IMO - International Maritime Organization

KBAL - Knutsen Ballast Water Treatment Technology

LNA - Low DNA content

LP - Low Pressure

MP - Medium Pressure

MPN - Most Probable Number

NMR - Nuclear Magnetic Resonance

PI - Propidium Iodine

PCR - Polymerase Chain Reaction

PMA - Propidium Monoazide

q-PCR - Quantitative Polymerase Chain Reaction

RAS - Recirculating Aquaculture System

RNA - Ribonucleic Acid

SSC - Side Scatter

USCG – United States Coast Guard

UV - Ultraviolet

VBNC - Viable But Non-Culturable

1. Introduction

From nutrient cycling to aquaculture, microorganisms are key players in aquatic ecosystems. However, potential risks such as spreading of non-indigenous species, infections due to microbial pathogens, and microbial blooms, are threatening water quality in water sources, water-based production systems, and water recipients. Detecting and monitoring microbes in such systems is essential to provide information on microbial water quality and the need for disinfection or other treatments. Flow cytometry-based techniques can be used to monitor microbial water quality efficiently.

1.1 When does microbial life end?

Disinfection of water to inactivate microorganisms comes with an implicit question: when is a microorganism considered "inactivated"? The definition of live and dead in microbiology is not as straightforward as one may think. Schrödinger questioned "What is life" with the purpose of understanding how cells are able to be "alive" (Schrödinger, 1944). Alive cells can, at the same time, go through different stages. Common terms to describe microbial cell states are "viability" and "vitality". A viable cell is capable of cell division and is metabolically active (Davey, 2011). It is also described as a cell able to form colonies in or on culture media. A vital cell is also metabolically active, but does not show growth in or on culture media, i.e. does not divide (Nebe-von-Caron et al., 2000). Both terms are inherently different and often misused as "viability" (Netuschil et al., 2014). As described later, most analysis methods inform about cell vitality, but not about viability and cell division (Netuschil et al., 2014). In this thesis, I have focused on "microbial vitality" as an indicator of life and activity. On the other hand, dead cells are difficult to define as there is no known limit between microbial life and death. They can be characterized either by a loss of nucleic acid, membrane integrity and functionality, and cellular energy or all three together leading to degradation (Davey, 2011).

In aquatic environments, cell physiology is adaptative and microorganisms can modulate their activity depending on the physical conditions and the nutrient availability to survive under environmental stress (Del Giorgio and Gasol, 2008). Cell

states can vary from highly active, to starvation, dormancy and dead. Highly active cells have active substrate uptake, metabolic activity and cell growth. Starvation is described as a state where nutrient concentration is "below the threshold of uptake capacities or of the minimum cell requirements" (Del Giorgio and Gasol, 2008). Dormancy state allows the persistence of cells with low or no metabolic activity to overcome unfavourable environmental conditions (Jones and Lennon, 2010). Microorganisms can enter dormancy for shorter or longer periods and form spores or cysts (Sussman and Douthit, 1973;Kaprelyants et al., 1993). This dormancy state can enhance dispersion in the environment, and even increase species resilience and persistence against perturbations (Mestre and Höfer, 2021). Another cell state, called "Viable But Non-Culturable" (VBNC) is defined by a temporarily loss of the cells ability to divide under certain stressful conditions, such as a change in temperature, nutrient concentration, salinity, osmotic pressure or pH (Colwell, 2000). Numerous bacterial species appear in natural environments in this VBNC state (Oliver, 2010). The various microbial cell states represent a challenge during analysis.

1.2 Viability

Viability (capacity to divide and form colonies) is commonly determined with three similar cultivation methods. Cultivation on solid media by pour plate or spread plate techniques determine the total cell concentration based on the colony forming units (CFU) counts (Breed, 1916). This standard method is used as quality control in many industries and within the health sector (Pfaller and Loreen, 1997;Dilbaghi and Sharma, 2007). The Most Probable Number method (MPN), is based on a serial dilution of the sample to estimate the number of viable cells (Colwell, 1979;Sutton, 2010). The Direct Viable Count (DVC) is another method to determine the concentration of viable microorganisms, where cells are incubated together with nalidixic acid, which inhibits DNA replication (Kogure et al., 1979;Yokomaku et al., 2000). Cells will thus grow by elongation but are not able to divide. The elongated cells are considered viable, whereas the non-elongated cells are metabolically inactive (Kogure et al., 1979).

Growth-based methods can be time consuming depending on the growth rate for each microorganism, and only a small fraction of environmental microbes can be cultivated under laboratory conditions (Allen et al., 2004;Hammes et al., 2011). To overcome this problem, measuring microbial vitality with molecular staining methods is a reliable alternative. For example, investigation of membrane permeability with the LIVE/DEAD staining kit is a widely used cell parameter for cell vitality (Boulos et al., 1999).

1.3 Vitality

Vitality can be described as the sum of parameters that constitute and define microbial life and death (Hammes et al., 2011). Those parameters include status of cellular components such as membrane integrity or DNA presence, but also metabolic processes like enzyme activity and Adenosine triphosphate (ATP) production. Determination of cell death can also be difficult as "there is no shortcut which would permit assessment of the moment of death" (Davey, 2011). To make it even more complex, cells can either repair themselves or be degraded, depending on external conditions. Braissant *et al*. reviewed current available assays for metabolic activity, of which most are bulk analysis and not adapted for environmental samples consisting of a multitude of different species (Braissant et al., 2020). ATP bulk measurements evaluate energy levels in cells, which reflects metabolic activity (Schneider and Gourse, 2004). The method has been used for several species, including *Escherichia coli*, *Bacillus sp.*, *Salmonella typhimurium,* and other environmental microbes. Metabolic activity can alternatively be evaluated with Redox assays, where tetrazolium salts (colourless compounds) are transported inside cells and reduced to a red or violet formazan derivate, and detected (Braissant et al., 2020). However, information and tests on bacteria are limited, and environmental samples should be interpreted carefully since activity in many bacteria may be present below the detection level (Grela et al., 2018;Braissant et al., 2020).

For environmental microbial analysis, autoradiography is a standard method to follow microbial metabolic activity through DNA replication or protein production

(Joint and Pomroy, 1987;Jellett et al., 1996;Sherr et al., 1999;Del Giorgio and Gasol, 2008;Yokokawa et al., 2012;Braissant et al., 2020). Incorporation of ^{3}H-Thymidine or ^{3}H-Leucine estimates DNA and protein production, respectively; as well as cell division frequency (Fuhrman and Azam, 1982). The method is specific to heterotrophic bacteria since autotrophs and protozoa do not incorporate ^{3}H-Thymidine. The incorporation of radioisotopes is a reliable method to follow cell activity, and can also be used in combination with Nuclear Magnetic Resonance (NMR) spectroscopy and other methods for metagenomic or metaproteomic analysis (Hatzenpichler et al., 2020).

Novel methodologies that focus on single cell metabolic activity are changing the way microbial vitality analysis can be carried out and interpreted. The application of single cell analysis for water quality monitoring may solve problems associated with enforcement of water quality regulations and their requirements. Such method allows us to evaluate individual cell life state within heterogenous microbial communities, something that cannot be explored in bulk analysis.

1.4 Areas of application for vitality methods within water quality monitoring

In marine aquatic environments, determination of vitality states is important within several areas of applications and to understand how microbes impact ecological processes (for example in the microbial loop and biogeochemical cycles). Microbes in aquatic ecosystems are important for maintaining nutrient fluxes. Although phytoplankton do not contribute to more than 1-2% of the total aquatic biomass they are responsible for half of the Earth's primary production (Falkowski, 1994). Phytoplankton participate in the regulation of dissolved organic carbon concentration (DOC) through photosynthesis in the water column and are part of nitrogen and phosphorus cycles (Ducklow et al., 2001;Falkowski et al., 2008). Heterotrophic bacteria are also involved in the nutrient cycling as they convert DOC into CO_2, making it available for phytoplankton consumption (Kirchman et al., 2009). Together, bacterial degradation and phytoplankton primary production regulate DOC in the oceans by forming a microbial loop (**Figure 1**) (Ducklow et al., 2001;Kirchman et al., 2009). High diversity among phytoplankton and bacteria in aquatic ecosystems is driven by

microbial competition and benefit balance in the food web (Pomeroy, 1974;Huisman and Weissing, 1999;Gibbons and Gilbert, 2015). Each microorganism has its own impact on ecological processes. Single cell analysis focusing on vitality by looking into metabolic activity, provides information of the active microbial fraction and how it may contribute to and influence ecological processes.

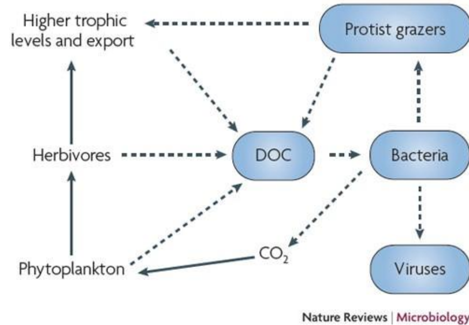

Figure 1: *Diagram of the food web (food chains in one ecosystem) with a focus on the microbial loop. The bacterial degradation of dissolved organic carbon (DOC) makes carbon available for phytoplankton consumption. Figure from Kirchman et al. 2009.*

Another area of application for vitality methods is to monitor microbial fish pathogens during aquaculture production. The potential presence of pathogens requires the use of water disinfection systems, with the subsequent need to monitor microbial vitality to ensure disinfection efficacity. Transport operations that involve movement of water from one place to another, such as well boats and ballast water in ships, can also disperse diseases and organisms when water is discharged in different regions. Such cases will also require compliance control after disinfection. For either case, with and without water disinfection, information around microbial vitality in water is necessary to understand and protect the environment and to improve biosafety in aquatic productions.

1.4.1 Aquaculture

In water bodies, pathogens and invasive species represent a risk for human and animal health (Cleaveland et al., 2007;Litchman, 2010). This is especially relevant for aquatic-based industries or those that use water resources in their production. Norway is currently the largest salmon producer in the world (1.388 million tons fish sold in 2020 (Directorate of Fischeries, 2021)), and is aiming to reach a total production volume of 5 million tons by 2050 (Norwegian Ministry of Fisheries and Coastal Affairs, 2012). To do so, optimal production systems that focus on animal welfare are essential, including appropriate living conditions for animals while preserving the natural environment as much as possible (Bentzon-Tilia et al., 2016). Bacterial communities associated with aquatic animals, including farmed animals, are fundamental to ensure growth performance to adult stage, convert waste compounds and ensure nutrient recycling in the water column during fish production (Bentzon-Tilia et al., 2016;Vadstein et al., 2018). At the same time, aquaculture farming requires control and monitoring of these bacterial communities. This has traditionally been done using antibiotics, egg disinfection, vaccination, and water disinfection. However, prophylactic antibiotics and water disinfection practices have shown poor performance and lack of reproducibility due to apparent unbalanced microbiota (De Schryver and Vadstein, 2014). Fish-microbial symbiosis represents an important factor against the emergence of pathogens and opportunistic microbes (Vadstein et al., 2004). Microbial probiotics have been of interest in aquaculture as they enhance fish immune systems, increase resistance against pathogens and promote animal growth (Langlois et al., 2021). Nevertheless, Norwegian aquaculture has been impacted by several bacterial diseases during the last decades, in both land-based and sea-based farms (Sommerset, 2020). For fresh water and marine environments, *Aeromonas salmonicida* subsp. *salmonicida* and V*ibrio anguillarum,* also called *Listonella anguillarum* have represented the main risks of disease (Dallaire-Dufresne et al., 2014;Hickey and Lee, 2018). In brackish waters, *Yersinia ruckeri* is known to cause the Red mouth enteric disease (Kumar et al., 2015). Sea water opportunists include *Moritella viscosa* and *Vibrio salmonicida* (Colquhoun et al., 2004;Kashulin et al., 2017). Finally, in fresh water *Flavobacterium psychrophilum* was and, still is one concerning pathogen

(Ekman et al., 1999;Norwegian Veterinary Institute, 2021). Other unwanted events, like phytoplankton blooms, have also been causing catastrophic loss for fish farmers in recent years. In 2019, Norwegian aquaculture was severely impacted with the loss of 8 million salmons in the Tromsø region (North Norway) due to the microalgae *Chrysochromulina leadbeateri* (Grann-Meyer, 2020;Engehagen et al., 2021). Similar episodes have also been observed in Norway with diverse species (e.g. *Gyrodinium aureolum*, *Prorocentrum minimum* and *Prymnesium parvum*) and in Chile with *Alexandrium catenella* blooms in the past (Dundas et al., 1989;S. et al., 1991;Mardones et al., 2015;Montes et al., 2018). Phytoplankton blooms are natural in the aquatic environment during spring, due to the upwelling of nutrients followed by growth of a high biomass over a short period (Smayda, 1997). Bacteria associated with high density blooms deplete dissolved oxygen via respiration during bloom decay (Hallegraeff, 2003), and lead to animal death (Møller and Riisgård, 2007). Climate change is impacting the phenomenon by increasing water temperatures causing the expansion of biogeographic locations and earlier blooms (Dale et al., 2006).

Determination of aquatic microbial vitality will provide useful information for a more sustainable aquaculture production and improved animal welfare. Other applications for vitality research could include the identification of active bacteria essential for waste conversion during production, prevention of microbial diseases via water disinfection and monitoring of potential algal blooms. As microbes are closely associated with animals, it is of interest to understand their beneficial interactions.

1.4.2 Ballast water

Water discharges, for example via ballast waters for ship stabilization, or by well boats for transport of live fish, need to be disinfected to prevent the spread of disease and non-indigenous species. The use of well-boats as transportation, either from land-based farms into sea cages, or from sea cages to the slaughter houses, can be a critical step in the spread of diseases (Murray et al., 2002;Norwegian Veterinary Institute, 2015). For example, furunculosis was imported from Scotland farms in the 80’s resulting in further spread of the disease in Norwegian farms (Egidius, 1987). On the other hand, well-boats contribute to biosafety as they are used for chemotherapeutants, freshwater- and physical treatments against sea lice (Overton et al., 2019). Similarly, the use of ballast

water follows strict regulation to prevent the spread of invasive species and diseases (International Maritime Organization, 2004). The release of alien microorganisms can impact the food web by competition and alter habitats (Crooks, 2002). Fisheries, aquaculture industry, and animal health can be affected by invasive species as well (Tsolaki and Diamadopoulos, 2010). *Aureococcus anophagefferens*, a toxic dinoflagellate responsible for red tides, is known for being transported in ballast water under a cyst resistant form (Doblin M. A. et al., 2004). Bacteria can colonize surfaces in ballast tanks during transport and form biofilms. They are more resistant to chemical disinfection and can release secondary biofilms to enhance dispersion (Costerton et al., 1999;Drake et al., 2007). A special concern has been the possible spread of pathogens toxic to humans (Cohen et al., 2012). An epidemic of *Vibrio cholerae* occurred in the Gulf of Mexico in the nineties as the strain was transported from North America by ships (McCarthy and Khambaty, 1994).

As our understanding of aquatic ecosystems widens, problems caused by human activity are also brought up. Regulations for environmental protection aim to control possible alterations by microorganisms. Single cell analysis of ballast water represents a reliable method to verify disinfection efficiency, regulation compliance and monitor cell vitality after treatments.

1.4.3 Importance of microbial vitality in regulations

Over the years, our knowledge on how infections and microbes spread has allowed the development of solutions to ensure good microbial water quality. Land-based and closed aquaculture farms, well-boats, and boats with ballast water, follow national and international regulations and use water disinfection systems to inactivate and/or remove microbes in their water systems. Norwegian regulations require land-based aquaculture farms to document and disinfect intake water and waste water from aquaculture-related activities (Forskrift om desinfeksjon av vann, akvakultur, FOR-2016-10-19-1217 (Norwegian Ministry of Fisheries and Coastal Affairs, 1997)). Well-boats used for transportation of live fish from land-based farms to sea cages or to slaughterhouses are also subject to new regulations (in 2015 and 2021) to prevent the spread of diseases (Norwegian Veterinary Institute, 2015;Norwegian Ministry of

Fisheries and Coastal Affairs, 2017). Since 2015, well-boats in Norway need to use an approved water disinfection system and record their activities (e.g. disinfection, water transfer, discharge) (Norwegian Veterinary Institute, 2015).

Regulations concerning ballast water treatment (The International Convention for the Control and Management of the Ships' Ballast Water and Sediments) were adopted by the International Maritime Organization (IMO) in 2004 (International Maritime Organization, 2004) and the U.S Coast guard (USCG) in 2012 (United States Coast Guard, 2012). The D-2 Ballast water performance standard and the Standards for Living Organisms in Ship's Ballast Water Discharged in U.S. Waters (International Maritime Organization, 2004;United States Coast Guard, 2012), describe the requirements that must be met, which are related to microorganisms loss of viability (loss of division) or inactivation (loss of vitality), respectively. For ballast waters, the D-2 standard focuses on human pathogens *E. coli, Vibrio cholerae* and intestinal *Enterococci*, whereas other potential pathogens are not mentioned at all (Lehtiniemi et al., 2015;Drillet, 2016).

To comply with the performance standards, different technologies have been developed and approved for water disinfection. Several physical and chemical water treatment systems are available on the market (Tsolaki and Diamadopoulos, 2010;Hess-Erga et al., 2019;Olsen et al., 2020;Sabo-Attwood et al., 2021). Physical disinfection often includes UV-irradiation and/or filtration, whereas chemical treatments include ozone and chlorine disinfection. It is common to combine different technologies in order to increase the effect, since no method is 100% efficient (Tsolaki and Diamadopoulos, 2010;Hess-Erga et al., 2019). These include combinations of UV and filtration (Wright et al., 2007), or cyclone and UV (Sutherland et al., 2001). Treatments using heat, ultrasound, or deoxygenation are also available, but are less efficient for disinfection of ballast water (Sayinli et al., 2022). Within aquaculture, UV and ozone treatments are preferred (Summerfelt et al., 2009;Martins et al., 2010).

UV-irradiation creates a photochemical reaction with biological components like DNA and RNA by producing pyrimidine dimers that alter the DNA structure and inhibit cell replication (Oguma et al., 2002;Hijnen et al., 2006;Coohill and Sagripanti, 2008). Proteins are also affected by UV-irradiation by inducing denaturation. The UV

spectrum is divided into three wavelength intervals: UVA (380-320nm), UVB (320-290nm), and UVC (290-190nm) (Coohill, 1996). Low pressure (LP) monochromatic UV lamps have a wavelength peak emission of 254nm (UVC), specific to target DNA/RNA structures. Medium pressure (MP) polychromatic UV lamps have a broader spectrum from 200 to 600nm (UVA, UVB, and UVC) to target all cell components, including DNA and proteins (Coohill and Sagripanti, 2008). A wide range of bacteria and phytoplankton are inactivated with low or high UV doses from LP and MP lamps (Hijnen et al., 2006;Gregg et al., 2009). UV efficiency can decrease due to particle shadowing, as observed for example in recirculating aquaculture systems (RAS), but can be compensated by increasing the UV doses (Sharrer et al., 2005;Gullian et al., 2012). Disinfection of microorganisms with UV-irradiation is a well-known method, and does not release toxic chemicals to the water (Tsolaki and Diamadopoulos, 2010).

Since USCG requires vitality analysis, new methods for single cell vitality detection need to be implemented for compliance control. However, standardization of analysis methods to enumerate vital microorganisms represent a challenge. External factors such as water temperature, salinity, pH, and microbial diversity may influence water treatments and analysis results (Sayinli et al., 2022). Additionally, other factors like microbial regrowth may occur even after water treatments, and thus cause microbial dispersion (Grob and Pollet, 2016;Hess-Erga et al., 2019). The distinction of microorganisms life state is challenging since all organisms present differences in growth and activity. Single cell methods represent an alternative approach to carry out analysis required for both IMO and USCG regulations.

1.5 Vitality stains for Flow Cytometry

Single cell analysis allows a deeper understanding of cell physiology (Emerson et al., 2017;Hatzenpichler et al., 2020). Unlike bulk measurement, single cell vitality analysis can be conducted with specific staining dyes for individual cellular components and processes (Nebe-von-Caron et al., 2000) before analyzing with microscopy and/or flow cytometry (FCM).

In the current work, I have focused on vitality stains that reflect cell states for phytoplankton and bacteria, with FCM analysis. CFDA-AM/SYTOX Blue (**Paper I**) were used to detect enzyme activity and membrane permeability, which is advantageous when detecting damaged cells, whereas BONCAT/SYBR Green (**Paper II, III**) focuses on protein synthesis and presence of DNA which is important characteristics when following *in situ* evolution of cell activity. These stains all determine cell vitality by different parameters. For BONCAT and CFDA-AM, I use the term "activity" instead of "vitality", as it is more representative of the cell parameter (protein synthesis and enzyme activity, respectively).

Detection of DNA content and analysis of membrane integrity are the most common approaches when using FCM to detect vitality (Muller and Nebe-von-Caron, 2010;Hammes et al., 2011;Buysschaert et al., 2016;Hoell et al., 2017). Stains like SYBR Green I, DAPI, and the SYTO stain family, all bind to the DNA of the cells (Muller and Nebe-von-Caron, 2010). DNA staining is fast and accurate but does not give information on cell activity, only on structural integrity. Membrane permeability stains like Propidium Iodine (PI) or the SYTOX stain family detect compromised membranes (Hammes et al., 2011), which gives information on cell damage. A drawback of PI/SYTO9 combination is the lack of a standard protocol, as the staining steps need to be adapted for each microorganism. Furthermore, several reports have showed difficulties in interpreting results from environmental microbial samples (Shi et al., 2007;Davey and Hexley, 2011). For example, autofluorescence from chlorophyll in phytoplankton overlaps with PI fluorescence, making this stain incompatible (Veldhuis et al., 2001). Enzyme activity is another reliable method to follow vitality in microbes (**Paper I**). Substrates like fluorescein diacetate (FDA) esterase and derivate

5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM) are cleaved by active enzymes and emit fluorescence allowing their detection (Díaz et al., 2010). FDA is known to have weak fluorescence for bacteria analysis, and problems with dye uptake have been observed in the past (Díaz et al., 2010). As the dye accumulates in cells with intact membranes, it is possible to combine it with membrane permeability stains (**Paper I**). Recent studies have shown the capacity of CFDA-AM in combination with permeable DNA dye SYTOX Blue to evaluate vitality of microalgae *Tetraselmis suescica* with FCM (Olsen et al., 2015;Olsen et al., 2016).

A new method developed for environmental microorganisms by Hatzenpichler (Hatzenpichler et al., 2014;Hatzenpichler and Orphan, 2015;Hatzenpichler et al., 2016) consist in incorporating an analogous amino acid into newly synthetized proteins. The method, which is called BioOrthogonal Non-Canonical Amino Acid Tagging (BONCAT), is based on the incorporation of L-Homopropargylglycine (HPG)/L-Azidohomoalanine (AHA), both methionine analogues, into proteins (**Paper II, III)**. A selective click reaction conjugates a modified fluorescent dye to the analogous amino acid making it detectable via fluorescence (Hatzenpichler et al., 2016). BONCAT has been used to study planktonic marine bacteria (Samo et al., 2014;Leizeaga et al., 2017;Sebastián et al., 2019); deep sea sediment bacteria and archaea (Hatzenpichler et al., 2016;Sebastián et al., 2019;Reichart et al., 2020), soil microbes (Couradeau et al., 2019), and marine virus-host interaction (Pasulka et al., 2018). The BONCAT method provides another side of vitality activity analysis and represents multiple possibilities for environmental analysis (Emerson et al., 2017;Hatzenpichler et al., 2020). Studies of the effect of temperature change, nutrients input, or nutrients limitation in an ecosystem may reveal modifications in microbial activity and impact on global processes like the microbial loop or carbon cycle.

2. Aims of the PhD thesis

The main aim of the thesis is to evaluate various methods for vitality measurements in combination with flow cytometry to provide high throughput tools for microbial water analysis. Each method was evaluated for its ability to determine microbial vitality after water disinfection.

The main objectives of my thesis are:

- To evaluate the use of single cell FCM methods to determine the vitality of phytoplankton with CFDA-AM/SYTOX Blue (**Paper I**) and bacteria with BONCAT/SYBR Green (**Paper II, III**).
- To evaluate BONCAT staining with FCM for vitality analysis of bacterial monoculture and environmental bacterial communities (**Paper II, III**).
- To use FCM methods to determine the efficiency of UV and heat treatment on microbes' vitality (**Paper I, III**) and regrowth (**Paper I**).

3. Phytoplankton and bacteria vitality analysis combined with Flow Cytometry

I have used vitality stains to detect enzyme activity and protein synthesis activity. These stains measure the level of activity according to the fluorescence levels; the terms "active" to "non-active" are used to describe alive to dying cells. CFDA-AM is an esterase substrate that detects enzyme activity in cells (**Paper I**). The staining is straight forward with short sample incubation time, similar to other vitality dyes like Rhodamine 123 (membrane potential) or CTC (respiratory activity) (Léonard et al., 2016). CFDA-AM provides information on cell activity at a given time. However, the enzyme could remain active in dying cells, giving therefore a false result and dead cell may be counted as live (Olsen et al., 2016). In order to reduce the number of false positives, CFDA-AM can be combined with SYTOX Blue, a DNA stain that only enters cells with compromised membranes (Olsen et al., 2016). In this way, one dye indicates the level of metabolic activity, while the other gives information about cell integrity. Analysis of *Tetraselmis suecica* showed that CFDA-AM/SYTOX Blue are good candidates to explore cell vitality (**Paper I**) (Olsen et al., 2016). Flow cytometry analysis of the dual stained cells reflected physiological characteristics from alive (active and intact cells), to dying (active cells with compromised membranes), and dead cells (non-active cells with compromised membranes). Additionally, the natural chlorophyll fluorescent emission from phytoplankton cells is used for total counts and to reduce background noise during FCM analysis. CFDA-AM/SYTOX Blue is a reliable double staining for vitality analysis on monocultures. Application to natural microbial communities will be necessary to verify the method capacity to distinguish cell state in natural samples. For bacterial analysis, alternative esterase substrate like carboxyfluorescein diacetate (cFDA) are preferred for analysis in combination with a total DNA stain (Buysschaert et al., 2016).

Protein synthesis in cells is another target for vitality analysis. In the current work, BONCAT was used to determine bacterial vitality (**Paper II, III**). The method detects newly synthetized protein via a click chemistry reaction. The action of incorporating new amino acids into proteins indicate an active protein synthesis machinery with

functioning ribosomes and available amino acids. The translation of RNA into proteins is used as an indicator of the cell capacity to survive, along with other indicators like membrane integrity, generation of energy, and growth (Breeuwer and Abee, 2000). L-Homopropargylglycine (HPG) analogous amino acid has the advantage of being incorporated as the first amino acid in most proteins, thus making a very large proportion of newly synthesised proteins detectable (~95%) (Dieterich et al., 2007). However, it is possible that non-active or incomplete proteins will have incorporated HGP and will thus be falsely counted as positives (cells with protein synthesis or active cells). There is no differentiation between active proteins that are produced in a healthy cell, and the ones that are malfunctioning in a dying cell. Since HPG will be the first amino acid incorporated, the error on vitality analysis can be quite large depending on the moment of cell analysis. BONCAT was here combined with a total DNA stain, SYBR Green, chosen for its capacity to analyse marine bacteria (**Paper II**). A clear advantage with this double staining, is the reduction of background signals for FCM, as well as the ease of identifying the bacterial population. Therefore, SYBR Green was used to determine cell counts and evaluate DNA degradation and damage after treatments. For bacteria, BONCAT/SYBR Green was adapted to follow protein synthesis in monocultures (*E. coli*; *Aeromonas salmonicida*; *Listonella anguillarum*; *Yersinia ruckeri* and *Bacillus cereus*) and in more complex natural sea water samples (**Paper II, III**). When using the BONCAT staining, cell loss occurred due to the several centrifugations during washing-steps. Some cell loss was observed for monocultures, but the problem can be higher for natural samples if they are not handled carefully (**Paper II**). However, the remaining cells that were analysed still represent a very large part of the population (90%), giving important information about the bacterial community as a whole (**Paper II**). Corresponding methods where you filter water samples with subsequent BONCAT staining, washing steps, and FCM analysis, also lead to cell loss (Couradeau et al., 2019). Cell loss can be considered a general problem for bacterial staining protocols and is not linked solely to the BONCAT method.

The two double staining techniques described, CFDA-AM/SYTOX Blue and BONCAT/SYBR Green, provide information about cell enzyme activity/permeability or protein synthesis activity/presence of DNA, respectively (**Paper I, II, III**), and both

can be analysed by FCM. Several authors have previously discussed the importance of combining stains in order to obtain accurate results on single cell vitality (Hewitt and Nebe-Von-Caron, 2004;Hammes et al., 2011;Léonard et al., 2016), and this has been confirmed in this thesis as well (**Paper I, II, III**). Combination of membrane permeability stains with esterase activity, respiratory activity, efflux pump and membrane potential are some examples of possible multi-parameter analysis with FCM (Léonard et al., 2016). Vitality analysis with multi-staining can provide information on cell damages and effects of treatments such as antibiotics or UV treatment (Villarino et al., 2000;Léonard et al., 2016). CFDA-AM/SYTOX Blue is a new combination of stains that provide information on cell vitality and give a reliable and full “picture” of cell activity and integrity. For BONCAT/SYBR Green, protein synthesis activity is the main parameter linked to cell vitality. However, to evaluate treatments targeting DNA, SYBR Green staining can differentiate heavily damaged DNA (Hammes et al., 2011). In the future, it would be interesting to expand the use of BONCAT/SYBR Green to phytoplankton and compare with CFDA-AM/SYTOX Blue stains to determine which double staining is the most efficient for vitality analysis.

The analysis of cell vitality with FCM has several advantages over traditional methods, like microscopy. Analysis is carried out with reduced running times (flow rates between 25-50 (for bacteria) and 1000 μL/min (for phytoplankton)) allowing for analyses of a high number of cells at once (**Paper I, II, III**). Secondly, the presence of different fluorescence detectors in the instrument makes multi staining possible; something that is more difficult with microscopy, as each fluorescence filter need to be used one at a time. Each flow cytometer can be equipped with a single or several lasers that expand the range of fluorescent dye colours that can be used and detected at once. It is possible to compensate the fluorescence emission of overlapping stains on the light spectrum (spill over correction). For example, CFDA-AM/SYTOX Blue are monitored together with the natural chlorophyll fluorescent emission from phytoplankton cells (**Paper I**) and different BONCAT stains (AF647, AF405, AF488) were tested along with various total DNA stains (LDS751 and SYBR Green) (**Paper II**). Detection of natural cell autofluorescence can be used in addition to stains. For example, a blue

emission was observed in unstained *Tetraselmis* cells after they were exposed to UV irradiations (**Paper I**). Thirdly, FCM can directly measure individual cells and their specific activity (**Paper I, II, III**), instead of bulk analysis following a population trend. However, the use of FCM can be problematic in specific cases, like for samples with low cell concentration where FCM running time will increase to record enough events. This implies that more background noise will be observed on FCM dot plots and can cause a shadowing-effect of the bacterial population signal (Emerson et al., 2017).

If we compare conventional methods like plate counts or PCR with vitality staining, we can note the advantages and drawbacks of each method. When cell viability cannot be observed, vitality stains can be an appropriate method to provide information of cell state. Plate count determines cells that can reproduce, but without looking into the cells that remain active but incapable of reproduction. *E. coli* cells showed activity (BONCAT) and integrity (with membrane permeability), but no reproduction over time (with plate counts) (**Paper III**). This cell state is similar to VBNC cells and cells under stress that are unable to grow on culture media (Oliver, 2005;Zhang et al., 2015;Schottroff et al., 2018). Here, vitality stains provided information when growth was not observed.

q-PCR is another traditional method, commonly used to detect and quantify specific species. However, q-PCR does not allow the distinction between the DNA from live and dead cells, or free DNA (Zeng et al., 2016); giving no information about vitality. Nevertheless, the combination of vitality dyes like ethidium monoazide (EMA) and propidium monoazide (PMA), with PCR may give information about vitality of specific species (Keer and Birch, 2003). EMA and PMA are membrane-impermeable reagents that bind to DNA when photoactivated, causing an inhibition of PCR amplification. In vital cells, the dye is excluded by the cell membrane, allowing PCR amplification (Cangelosi and Meschke, 2014). Pre-treatment steps to remove DNA from dead cells or from cells with compromised membranes are, however, causing problems as treatments fail to completely remove dead cell DNA (Codony et al., 2020). Moreover, the method is not adapted to follow the entirety of a natural community. Some properties of this type of samples (e.g. turbidity, salt content) can interfere with the method and give false positives (Emerson et al., 2017). The combination of FCM

and cell sorting with subsequent PCR analysis could be a way forward to obtain cell identification and information on cells states, especially for community analysis. Combination of BONCAT with cell sorting and subsequent identification will provide information on unculturable bacterial species and expand our knowledge of rare microbes. It is of great interest to add the concept of vitality to species identification.

4. Vitality of bacterial monocultures and bacterial aquatic communities

I applied BONCAT/SYBR Green staining to follow protein synthesis for bacterial monocultures of *Escherichia coli*, *Yersinia ruckeri*, *Aeromonas salmonicida*, *Bacillus cereus*, and *Listonella anguilarrum*, but also for more complex samples from natural marine bacterial communities (**Paper II, III**). Monocultures and natural communities have different specific physiological characteristics and vitality levels that can influence vitality analysis. This difference of vitality observed between bacterial monocultures and natural communities can be explained by different parameters like nutrients concentration, presence of other organisms (e.g. viruses), species diversity, and/or presence of particles and aggregates. In **Paper II and III**, I used monoculture of indicator species *E. coli* to develop the staining protocol. BONCAT/SYBR Green staining revealed a continuous and stable protein production and the presence of DNA in *E. coli* over time. The rapid protein production observed for monocultures is typical for cells in exponential phase and at optimized growth conditions, and hardly any non-active cells were observed. In **Paper II** and **III**, two populations were observed on FCM dot plot in *E. coli* positive controls identified as High and Low DNA content (HNA and LNA, respectively). During experiments, it was sometimes possible to observe 4 populations, where two HNA and two LNA populations showed different protein synthesis levels (**Paper III,** Figure 2). The variation in protein synthesis levels can be due to the change of conditions (from original cell culture to cell suspension in 1X PBS and centrifugations) and a subsequent modification of cell metabolism due to nutrient input. Alternatively, it is possible that those populations represent the different states of cell division (mother/daughter cells) with different levels of protein synthesis and DNA content. Cells aim at maintaining homeostasis and cell size during growth in steady-state condition. This implies that protein content will fluctuate to stabilize homeostasis during cell growth and division (Kiviet et al., 2014). It remains unclear if the observations in **Paper III** are related to homeostasis balance and supplementary studies are necessary to confirm this.

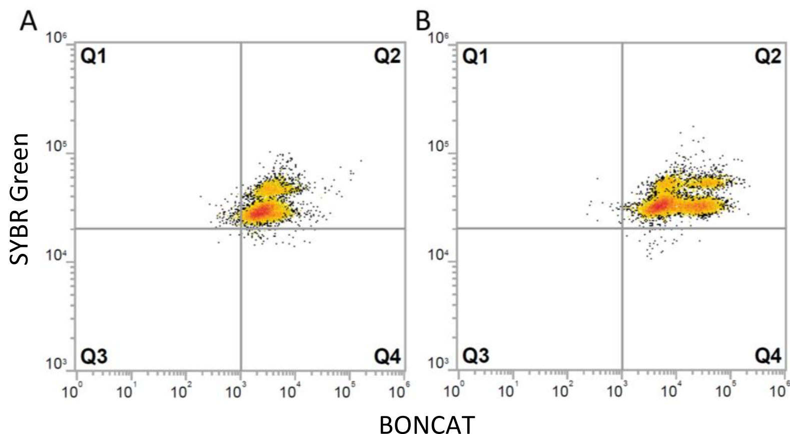

Figure 2: FCM dot plot of E. coli stained with BONCAT/SYBR Green as seen with SYBR Green fluorescence at the y-axis and BONCAT fluorescence at the x-axis. Each dot represents a single cell, and a colour gradient shows the cell density (red = most dense/highest abundance). Each plot is divided into four quadrants (Q1 to Q4) according to fluorescence levels. Cells with fluorescence intensity below $2x10^4$ for SYBR Green, and below 10^3 for BONCAT are not fluorescent. A) Q2: Two active populations, whereof one with high DNA content (HNA) and one with low DNA content (LNA) can be observed. B) Q2: Four active populations including two HNA and two LNA populations that varies in their protein production.

BONCAT is a suitable methodology for rapid detection of protein production in single bacterial cells. However, some surprising results are presented in **Paper III** with dead control samples from bacteria monocultures giving a false positive signal indicating protein production. The samples were killed with formaldehyde fixation 24h before L-Homopropargylglycine (HPG) was added. Different bacterial strains were tested (*E. coli*, *Y. ruckeri*, *A. salmonicida*, *B. cereus*, and *L. anguilarrum*, and samples from natural marine bacterial communities), and contrasting results were observed. Three out of five bacteria showed a false positive activity with BONCAT when cells were killed with formaldehyde. The question remains as to why some bacteria retain the HPG amino acid even though they are considered dead. The phenomenon was not observed for natural marine bacterial cultures. The wide diversity of bacteria in seawater may explain the absence of reaction to HPG and formaldehyde. Other fixation methods to prepare dead bacterial samples like isopropanol or acetic acid, showed a decrease of protein production in cells compared to controls (**Paper III**).

This open the question if HPG is a suitable molecule for BONCAT and whether another fixation method should be used instead for dead controls. In addition, recent research on BONCAT and HPG (Personal communication from Lotta A. Andor, June 2022) showed an antibacterial effect of HPG. HPG concentration from 5.6 to 90 µM stopped *E. coli* growth and significantly reduced growth during exponential phase. The other analogous amino acid used for BONCAT, AHA, did not influence growth during incubation with concentrations up to 9000 µM. Earlier studies of HPG have shown that the amino acid does not significantly influence cell metabolism in *E. coli* (Dieterich et al., 2006;Hatzenpichler et al., 2014;Steward et al., 2020). Those conflicting results imply that more research on analogous amino acids is necessary to remove any bias on the method.

Natural bacterial communities most often contain one apparent active and one non-active population compared to monocultures grown under optimum growth conditions (Del Giorgio and Gasol, 2008). The marine samples analysed in my studies had a higher number of cells with low activity than the *E. coli* cultures where the protein synthesis activity showed a statistical normal distribution when grown under optimal nutrient conditions (**Paper II**). BONCAT/SYBR Green gives information on single cell activity for heterotrophic bacterial community instead of a bulk and average activity. This is advantageous as it is possible to select and analyse cells according to their level of activity. The identification of cells in different states (from active to non-active) may help to understand community dynamic and possible mechanisms involved (Del Giorgio and Gasol, 2008). More precisely, it is possible to follow population activity shift due to factors like grazing or viral lysis, change in nutrient concentration or nutrient depletion, and temperature change. Also, as only a certain proportion of marine bacteria are shown active (20-50% (Kirchman, 2016); 10-30% (**Paper II and III**)), it is of interest to explore more precisely the limit between non-active and active cells to uncover cells with minimal activity and the ones with maximal activity. The difference is important as cells may have different metabolism, survival mechanisms, and energy uses. Exploring bacterial activity and distribution will provide more information to understand ecology theories like “Killing the Winner” (Thingstad and Lignell, 1997;Thingstad, 2000) which describes how the microbial communities are

structured as a consequence of trade-off between competition and defence, where we find only a few highly active and competitive microbes compared to the many resistant microbes with low activity. To further expand the use of BONCAT, it is possible to search for links between bacterial activity and specific microbial characteristics (e.g metabolisms and pathogenicity). Hatzenpichler et al. identified active anaerobic methane-oxidizing bacteria in deep sea sediments and discovered interaction with an anaerobic methane-oxidizing archaea consortia unknown until now (Hatzenpichler et al., 2016).

For marine water analysis, setting the quadrants is one of the difficulties when using FCM, as some autofluorescent signals from phyto-pico plankton may appears along BONCAT fluorescence. BONCAT is a relatively “new” methodology for analysing environmental microbes and it has so far only been applied to a limited number of species (from sediments, soil or seawater) (Hatzenpichler et al., 2014;Samo et al., 2014;Hatzenpichler and Orphan, 2015;Hatzenpichler et al., 2016;Leizeaga et al., 2017;Pasulka et al., 2018;Couradeau et al., 2019;Sebastián et al., 2019;Lindivat et al., 2020;Reichart et al., 2020;Steward et al., 2020;Lindivat et al., 2021). Analysis of natural samples therefore requires careful interpretations. For example, some cells may not incorporate HPG due to their physiological characteristics and uptake mechanisms (Hatzenpichler et al., 2014). Also, differences in incubation conditions from sampling locations to laboratory conditions may promote the growth of some individuals over others. However, in this study it is unlikely that this affected bacterial growth as no major boost of protein synthesis or increase of the active fraction was observed during the experiments (**Paper II**).

5. Microbial vitality after water disinfection

In some cases, microbial vitality analysis needs to meet specific requirements. Type approval of ballast water treatment systems for example calls for a methodology that allows for enumeration of vital organisms of different size-classes (International Maritime Organization, 2004;Hess-Erga et al., 2019). In my current work I selected vitality stains CFDA-AM/SYTOX Blue and BONCAT/SYBR Green combined with FCM analysis for the purpose of exploring microbial activity after UV treatment of *T. suecica* (**Paper I**) and UV and heat treatment of *E. coli* and a mixed community of seawater bacteria (**Paper III**). UV treatment is frequently used in ballast water treatment systems as well as in aquaculture for disinfection of intake and wastewater. Heat treatment, on the other hand, is not commonly used due to the high cost, high energy consumption, and long treatment time (Sayinli et al., 2022). However, heat treatment was chosen due to its different effect on cells compared to UV irradiation.

After irradiation with a MP UV-lamp and staining with CFDA-AM/SYTOX Blue, *T. suecica* dying cells displayed damaged membranes (SYTOX Blue fluorescence) with a decrease of enzyme activity (CFDA-AM). The results revealed a dose-response effect with increasing damages with higher UV-doses. CFDA-AM/SYTOX Blue and FCM is therefore suitable to detect phytoplankton death after UV treatment (**Paper I**) (Olsen et al., 2016). Some phytoplankton cells survived several days after treatment and, depending on the disinfection dose, a variable fraction of the cells recovered when in favourable conditions. Cell regrowth after disinfection treatment is problematic for ballast water, aquaculture, and water downstream processes as opportunistic microorganisms can recolonize water and be a source of risks as invader or pathogens (Hess-Erga et al., 2010). Regrowth was observed for *T. suecica* cells treated with UV doses under 200mJ/cm^2, with an increase of cell concentration from 10^4 cells/mL to 1.8x10^5 cells/mL (100mJ/cm^2) and 3x10^4 cells/mL (200mJ/cm^2) (**Paper I**). However, in the case of water release after disinfection, natural microbial communities are resilient and will return to a stable state as the surviving cells may not be able to compete or will not adapt to the new environment (Gomez-Alvarez et al., 2016). Additionally, microbial components from damaged or dead cells can represent a

problem when released into the environment. Cell debris will provide nutrients for other organisms to grow and release extracellular DNA (Allocati et al., 2015). DNA from dead cells can persist in water for a year and provide genetic material for bacterial transformation (Young et al., 2007;Allocati et al., 2015). Similarly, enzymes like proteases can be active up to 72h after cell death and can persist up to 96h in lake waters (Kiersztyn et al., 2012) or in heat treated activated sludges (Yan et al., 2008). This raises concerns for water quality after ballast water- or aquaculture discharge as DNA and microbial activity will be present even after cell death. CFDA-AM/SYTOX Blue combined with FCM is a good method for analysis of indicator organisms in the 10-50µm size category for type approval of ballast water treatment systems. The double staining has been used for efficiency testing of Knutsen Ballast Water Treatment Technology (KBAL) (Olsen et al., 2020). KBAL system uses a sudden pressure drop coupled with UV irradiation to disintegrate and inactivate cells. The study showed consistent *T. suecica* vitality results between laboratory UV treatment tests and KBAL treatments. Previous studies have stated the usefulness and good quality of FCM analysis for water quality (Hammes and Egli, 2010;Hoell et al., 2017;Cheswick et al., 2019;Safford and Bischel, 2019). However, despite being a successful and easily applicable method, the use of FCM for monitoring programs remains limited. Switzerland is the only country that includes FCM in compliance control analysis for drinking water since 2012 (Safford and Bischel, 2019). The reasons for this can be lack of knowledge in FCM analysis combined with the cost of the instrument. FCM combined with vitality stains is a good alternative to the traditional methods in monitoring programs and should be included to increase the toolbox.

BONCAT/SYBR Green was used on *E. coli* and seawater bacteria from natural communities to determine vitality after UV irradiation and heat treatment (**Paper III**). The cell structures were clearly affected after treatment (confirmed with a modification of the Side Scatter/Forward scatter (SSC/FSC) signal on FCM plots); yet the incubation time was not sufficient to observe more severe effects on protein synthesis. For heat treatment, a faster DNA degradation was observed in *E. coli* compared to UV treatment. SYBR Green is a good indicator for DNA degradation in this case, as it was possible to observe a decrease of DNA fluorescence in treated cells. However, only

extensive DNA damages can be monitored with SYBR Green (Hammes et al., 2011). Compared to UV irradiation, heat treatment had a more immediate effect on protein production and DNA integrity.

Seawater bacteria were mostly inactive after UV treatment (below 5% of active bacteria for dose 100 and 200 mJ/cm^2) (**Paper III**). Other studies of marine bacteria exposed to UV-B radiation revealed a similar decrease in protein production (Arrieta et al., 2000). For heat treatment, all cells were inactivated (**Paper III**). BONCAT was able to identify the proportion of active and inactive cells after both UV and heat treatment (**Paper III**). Yet, we cannot distinguish between inactive or dormant cells (**Paper III**). Supplementary experiments and additional conditions (e.g. different nutrient concentration, effect of particles) are necessary to fully explore BONCAT/SYBR Green capacity to determine bacterial activity in any conditions.

As BONCAT will detect any protein containing HPG, even damaged or incomplete proteins will be detected. To fully observe dying and dead cells with BONCAT, it is necessary to stain and record protein synthesis for a longer period than what I did (24h). In addition, modifying the incubation time with HPG would be beneficial to observe a decrease of protein synthesis in damaged cells. It is possible that extended incubation may influence vitality analysis as cells will have more time to incorporate HPG. The calculation of HPG incorporation rate would be interesting to confirm it. Samo *et al.* used the top 10% isolate mean method to determine protein production rate with the fluorescent intensity of single cells after BONCAT (Samo et al., 2014). However, many factors such as cell numbers, bacterial diversity, biomass, growth rate, and single cell fluorescence, make the protein production rate a difficult factor to determine. FCM analysis of BONCAT cells to determine protein production rate will require tests on single species. The aim is to obtain a general formula of protein synthesis production rate that can be used for community analysis and various growth rates. It is important to be aware that different FCM instruments may display differences in fluorescence levels due to the variations in detectors and electronics. BONCAT/SYBR Green is reliable to identify active cells, but additional applications, like determining protein production rate or growth rate, is challenging for now. The application of BONCAT to microbial vitality analysis after treatment is not entirely optimized. Further experiments

are needed to determine if protein synthesis is adapted for this purpose. However, as shown previously in several works, BONCAT is adapted to follow vitality in aquatic microbes (**Paper II**) (Samo et al., 2014;Leizeaga et al., 2017;Sebastián et al., 2019). In addition, BONCAT can be applied for antibacterial molecules testing. With the current challenge of antibiotic resistance, testing the effects of new antimicrobial substances on bacterial cells has become a priority. BONCAT is a candidate to be added to the catalogue of vitality methods that characterize antimicrobial treatments at single cell level in the future (Léonard et al., 2016).

6. Future research

Fluorescent vitality stains coupled with FCM analysis represent useful means to obtain microbial vitality information. CFDA-AM/SYTOX Blue and BONCAT/SYBR Green represent new vitality staining methods for microbial water quality analysis. By providing information on advantages, drawbacks, and efficiency for each method, this thesis aimed to develop and expand the range of single cell vitality analysis for FCM.

CFDA-AM/SYTOX Blue and FCM analysis is reliable for vitality and regrowth analysis of *T. suecica* cells after UV treatments (**Paper I**). One application of the method is the monitoring of microorganisms in the size range 10-50 µm according to the IMO and USCG regulations for microbial quality after ballast water discharge.

As BONCAT combined with FCM is a new method, it has not been extensively used yet (**Paper II, III**). There is still more work required for optimization like the use of alternative analogous amino acid. However, future research should focus on the potential uses of BONCAT and FCM to further understand microbial activity. The next step will be the combination of vitality analysis with species identification methods like Fluorescence *in situ* Hybridization (FISH), followed by cell sorting. This combination will allow to not only detect specific bacteria according to their species or characteristics (e.g. genes related to antimicrobial activity, genes involved in carbon metabolisms etc), but also to know their vitality in any conditions. New detection probes with branched fluorescence like PrimeFlow (Thermofischer Scientific, USA) increase the fluorescence signal during FCM detection, thus making the method a good candidate to detect specific cells in complex sample and to combine with BONCAT.

The application of vitality stains in aquaculture and ballast water quality monitoring will enhance our knowledge about the possible interactions between microbes and aquatic animals and their impact in new environments. A natural progression of this work will be to focus on aquaculture and the detection of pathogens and/or beneficial microorganisms and their vitality to provide accurate microbial water quality assessment for this industry. A recent study has shown the importance of bacteria associated with aquatic animals as source of rare microbial taxa (Troussellier et al., 2017). The study demonstrated the abundance evolution of rare microbial taxa under

natural growth conditions, and study the influence of aquatic animals on the active fraction of microbes (Troussellier et al., 2017). Focus on microbial vitality will allow us to have a better understanding of aquatic microbes and the aquatic environment.

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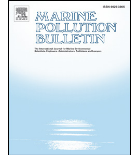

Incubation in light versus dark affects the vitality of UV-irradiated *Tetraselmis suecica* differently: A flow cytometric study

Ranveig O. Olsen[a,*], Mathilde Lindivat[a], Aud Larsen[b], Gunnar Thuestad[a], Ingunn Alne Hoell[a]

[a] Western Norway University of Applied Science, Bjoernsonsgt. 45, 5528 Haugesund, Norway
[b] Uni Research Environment and Hjort Centre for Marine Ecosystem Dynamics, 5006 Bergen, Norway

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ABSTRACT

In this study, we used flow cytometry to examine how incubation in dark versus light affects the vitality and viability of UV-irradiated *Tetraselmis suecica*. High UV doses (300 and 400 mJ/cm^2) affected the esterase activity, membrane permeability, and chlorophyll content more when the subsequent incubation took place in light. For non- or low UV dose (100 and 200 mJ/cm^2)-treated cells, incubation in light resulted in cell regrowth as compared to incubation in dark. Damaged cells (enzymatically active but with permeable membranes) did not recover when incubated under light or dark conditions.

Exposure to light reduces the evaluation time of any given ballast water treatment, as viable cells will be detected at an earlier stage and the vitality is more affected. When evaluating the performance of UV-based ballast water treatment systems (BWTS), these results can be useful for type approval using *T. suecica* as a test organism in the test regime.

1. Introduction

Ballast water is used to maintain stability and maneuverability of ships, and the transfer of non-native, possibly invasive species, into ship's ballast water is of global concern (Bax et al., 2003; Occhipinti-Ambrogi, 2007). To minimize the maritime industry's environmental footprint caused by ballast water, global and national frameworks have been developed. The International Maritime Organization (IMO) and the U.S. Coast Guard (USCG) adopted standards, in 2017 and 2012, respectively, specifying the same allowed limit concentrations of indicator bacteria species and different size categories of organisms in ballast water discharge. To comply with the standards, ships have to install onboard ballast water treatment systems (BWTS) to treat the water at uptake, discharge, and/or during the voyage (David and Gollasch, 2015; Lloyd's Register Marine, 2019). Numerous BWTS have been approved and are commercially available, and the majority of technologies rely on physical or chemical modes of action for disinfection (David and Gollasch, 2015; Lloyd's Register Marine, 2017, 2019; Shannon et al., 2008; Werschkun et al., 2014; Werschkun et al., 2012). Yet, there are no BWTS that can guarantee 100% eradication of all organisms at discharge (Batista et al., 2017; Grob and Pollet, 2016). An analysis with the aim of detecting remaining organisms in the treated ballast water is necessary to evaluate the treatment efficiency during approval of BWTS and for compliance testing of ballast water at discharge (International Maritime Organization, 2013). For organisms within the size fractions ≥ 10– $< 50\,\mu$m and $\geq 50\,\mu$m, IMO requires enumeration and detection of viability (ability to reproduce) for ballast water approval (International Maritime Organization, 2004). USCG, on the other hand, requires enumeration and detection of cellular vitality (ability to live) of organisms in the ≥ 10– $< 50\,\mu$m size fraction (U.S. Environmental Protection Agency, 2010),

Ultraviolet (UV) radiation is a treatment technique involving exposure to either low-pressure (LP) or medium-pressure (MP) UV lamps and is used in > 30% of present-day BWTS (David and Gollasch, 2015; Delacroix et al., 2013; Lloyd's Register Marine, 2017, 2019; Werschkun et al., 2012). LP UV lamps emit almost all energy at 254 nm (UV-C) as monochromatic light, which can cause RNA/DNA damages, possibly inhibiting replication and transcription (G. B. Sancar, 1990; Weber, 2005). Vitality stains such as fluorescein diacetate (FDA) and 5-chloromethylfluorescein diacetate (CFDA) required for enumeration and detection in ballast water analysis by USCG (U.S. Environmental Protection Agency, 2010) do not detect DNA damages. MP UV lamps (emitting UV-A, -B, and -C light) may additionally cause formation of reactive oxygen species (ROS), thereby causing oxidative damage to lipids, proteins, and DNA (Bosshard et al., 2010; Kalisvaart, 2001; Kottuparambil et al., 2012; B. Kramer and Muranyi, 2014; Ana L. Santos et al., 2012; Ana L Santos et al., 2013). Loss of membrane potential induced by UV light can cause loss of efflux pump activity (Berney

* Corresponding author.
E-mail address: ranveig.olsen@hvl.no (R.O. Olsen).

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et al., 2006; Bosshard et al., 2009; B. Kramer and Muranyi, 2014). Additionally, UV-B radiation can affect key components in photosynthesis (Fiscus and Booker, 1995; Holzinger and Lütz, 2006; Kottuparambil et al., 2012), and radiation at 280–400 nm (solar UV radiation) can inhibit growth and photosynthesis (Gao et al., 2007; Heraud and Beardall, 2000) as well as harm cell membrane and pigment complexes (G. F. Kramer et al., 1991; Ma and Gao, 2010). UV-B radiation (280–315 nm) caused decrease in growth rate, chlorophyll *a* and carotenoid content of *Tetraselmis* spp. (Yu et al., 2004; Zhang et al., 2005). Motility and photosynthesis of the same phytoplankton species were impaired by enhanced solar UV radiation (Ma et al., 2012), and MP UV radiation caused permeable membranes and loss of esterase activity (Olsen et al., 2016a; Olsen et al., 2015; Olsen et al., 2016b).

Phytoplankton are photosynthetic organisms that rely on solar radiation for photosynthesis, but exposure to strong light can also cause photoinhibition (Andersson and Aro, 2001; Aro et al., 1993; Häder et al., 1998; Murata et al., 2007; Powles, 1984) or formation of various ROS (Niyogi, 1999). Several green algae have adapted to high levels of solar UV irradiation, possibly by efficient avoidance mechanisms such as UV-absorbing substances (pigments) and repair of UV-induced damages (Andersson and Aro, 2001; Aro et al., 1993; Govindjee and Papageorgiou, 2004; Rastogi et al., 2010; Sinha and Häder, 2002; Sinha et al., 1998). UV-induced DNA damages can be repaired in the presence (photoreactivation) or absence (dark repair) of light (A. Sancar and Sancar, 1988; Sinha and Häder, 2002). UV damages like increased cellular membrane permeability (Olsen et al., 2016a) can be reversed, that is, a temporary condition (Davey and Hexley, 2011; Duffy et al., 2000; Shi et al., 2007). Darkness will naturally also affect the vitality of photosynthesizing organisms with time (Berges and Falkowski, 1998; Jochem, 1999), although phytoplankton can adapt to prolonged periods of darkness retaining their photosynthesizing capabilities, by reducing their metabolism to a lower activity level (Carney et al., 2011; Jochem, 1999). Previous studies demonstrated that non-irradiated *T. suecica* cells incubated in darkness for 15–25 days and re-exposed to light were vital and viable, and this was also the case for cells pre-treated with MP UV doses of 100 and 200 mJ/cm^2 and incubated in the dark for a few days (Olsen et al., 2016a; Olsen et al., 2015). However, the numbers of viable cells were reduced during dark incubation both for the non-irradiated and for the UV-irradiated samples. UV treatment and subsequent incubation in dark for 5 days caused more than a 90% reduction in viable *Tisochrysis lutea* (Romero-Martinez et al., 2016). When the cells were re-exposed to light, however, growth was promoted in all samples. As organisms can survive unfavorable conditions and regrow after repair, this can affect the BWTS disinfection efficiency and the analysis for BWTS approval and compliance testing of ballast water.

To determine the vitality of UV-irradiated cells, we have previously developed a flow cytometry (FCM) protocol taking advantage of dual staining by the esterase substrate 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM) and SYTOX Blue. The FCM protocol stains metabolically active and membrane-permeable cells, respectively, and by that differentiating the phytoplankton *T. suecica* into live, damaged, and dead cells (Olsen et al., 2016a). We herein use this protocol to examine how incubation in dark versus light affects UV-irradiated *T. suecica*. We aimed at answering the following questions:

1) Does incubation in dark affect esterase activity, cell permeability, and chlorophyll content (vitality) of *T. suecica* differently from incubation in light?
2) Can the vitality of damaged *T. suecica* cells be reversed when incubated in light, so that the cells can recover again?
3) Is the FCM protocol suitable for regrowth (viability) studies?
4) Can our FCM protocol and incubation regimes give recommendations regarding IMO and USCG BWTS regulations?

2. Material and method

2.1. Experimental setup

The phytoplankton *T. suecica* was chosen as a test organism, as it can be included in the test water as a representative for the 10–50 µm size group for land-based type approval of BWTS (D'Agostino et al., 2015). Additionally, this alga is considered as a model organism for UV damage experiments (Hull et al., 2017; Liu et al., 2016; Lundgreen et al., 2019; Olsen et al., 2016a). *T. suecica* strain K-0297 was obtained from the Scandinavian Culture Collection of Algae and Protozoa (University of Copenhagen, Denmark). It was cultured in 36 g kg^{-1} artificial sea water (ASW) (Marine SeaSalt); then, 0.12% Substral (The Scotts Company (Nordic)) was added and incubated in light at 36 W m^{-2} (corresponding to 173 µmol m^{-2} s^{-1} of photosynthetically active radiation (PAR) photons) at 15 °C and 1.7 cm orbital shaking at 100 rpm. The culture was diluted with growth medium (ASW added 0.12% Substral) to a concentration of 10^4 cells/ml before irradiation, monitored by FCM.

For UV exposure, a collimated medium-pressure (MP) 800 W UV lamp (BestUV) with a polychromatic (200–400 nm) mercury lamp was used (Olsen et al., 2015). The UV-C intensity was 0.525 mW cm^{-2}, and the weighted average germicidal factor, based on the absorbance spectrum of DNA from *Bacillus subtilis* (Chen et al., 2009), was 0.5799.

Forty-five-milliliter aliquots of diluted *T. suecica* were irradiated in Petri dishes (6.7 cm in diameter) under mixing with a magnetic stir bar (150 rpm) at room

temperature (RT). The exposure times were 178, 356, 533, and 711 s for UV doses of 100, 200, 300, and 400 mJ/cm^2, respectively. At each UV dose, three parallel samples were prepared. UV doses of > 400 mJ/cm^2 were not applied, as previous FCM studies showed that *T. suecica* UV irradiated at 400 mJ/cm^2 were permanently inactivated (Olsen et al., 2016a; Olsen et al., 2016b). Each sample was split after irradiation, and 22 ml was subsequently transferred into two 50 ml Falcon conical centrifuge tubes (Fisher Scientific). Nonirradiated cells (2 × 22 ml) were also transferred into Falcon tubes. Half of the split samples were wrapped in aluminum foil and incubated with loosened lids at 15 °C in the dark (i.e., dark incubation conditions), and the other half of the samples were incubated with loosened lids at 15 °C in light (36 W m^{-2}). The tubes were incubated up to 7 days without movement; however, samples were gently mixed at collection and subsequently analyzed at 2 h and 1, 2, 3, 4, and 7 days. An overview of the setup for the experiment is given in Fig. 1.

2.2. FCM analysis

The cells were dually stained with the esterase substrate CFDA-AM (C1354, Thermo Fisher Scientific, USA) and SYTOX Blue Dead Cell Stain (S34857, Thermo Fisher Scientific, USA) before FCM analyses, as previously described (Olsen et al., 2016a) with the only difference using 1 mM SYTOX Blue instead of 0.3 mM working solutions.

FCM analysis was performed on an Attune Acoustic Focusing Cytometer (Thermo Fisher Scientific) as previously described (Olsen et al., 2016a), with a threshold set on 10,000 for the BL3 detector (640LP). Due to an observed decrease in red autofluorescence for samples irradiated with 300 and 400 mJ/cm^2 and incubated in light, the threshold was reduced to 1000 on days 4 and 7 (only for these samples). One milliliter of each sample was analyzed at a flow rate of 1000 µl min^{-1} at standard sensitivity. As control samples, both unstained and dual-stained nonirradiated cells were used.

The initial sample volume was 22 ml. A total of 1.5 ml of each sample was taken out for dual staining every day, and 1.5 ml was removed on days 3, 4, and 7 for unstained samples, leaving 8 ml at the end of sampling.

The FCM signals from nonirradiated and UV-irradiated *T. suecica* cells dual-stained with CFDA-AM and SYTOX Blue, incubated in light or

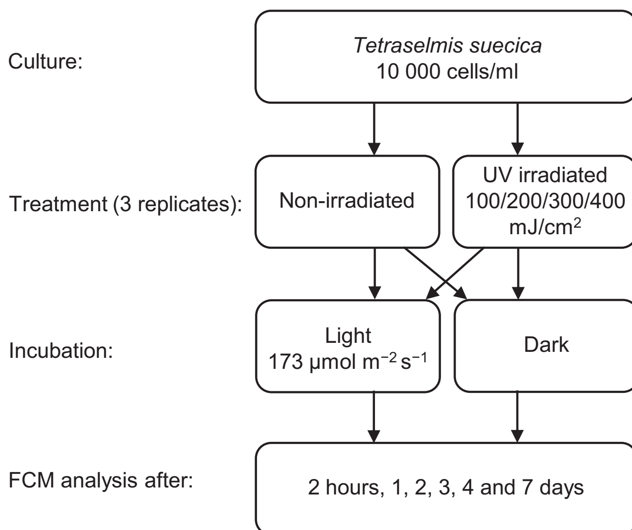

Fig. 1. Experimental set-up showed by a flow diagram.

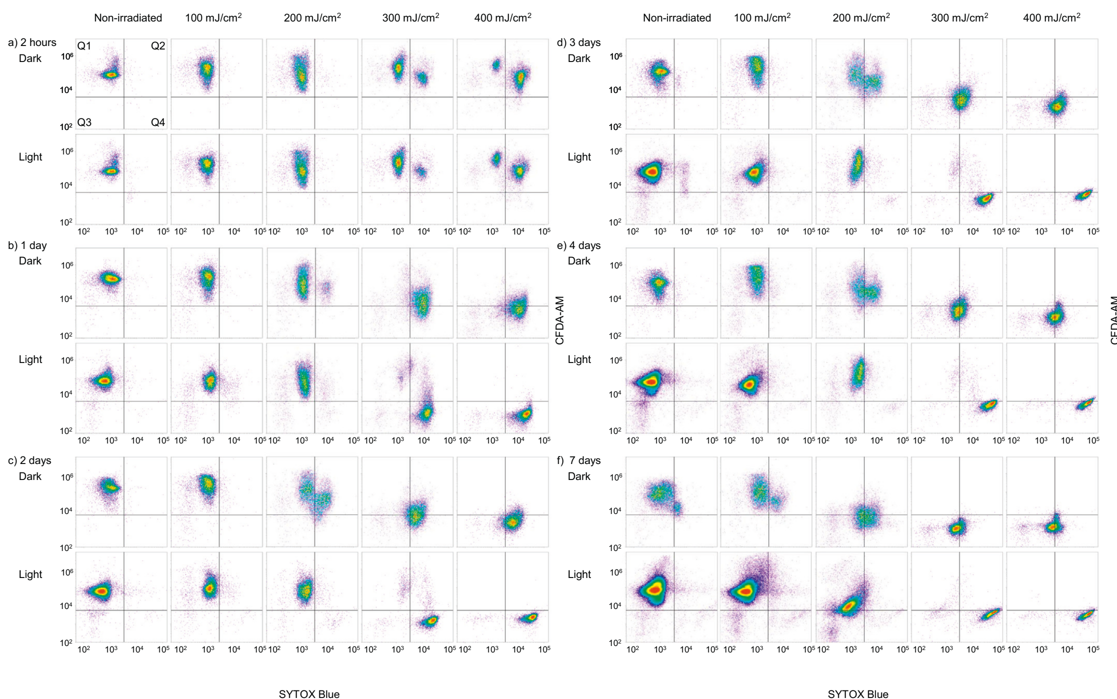

Fig. 2. SYTOX blue.

dark, are presented as dot plots (Fig. 2). The dot plots are separated into 4 quadrants (Q) by a vertical and a horizontal line, based on their green and blue fluorescence intensity (Olsen et al., 2016a). The quadrants reflect various physiological cellular characteristics (esterase activity and membrane permeability), and thus vitality. The cells in Q1 have high esterase activity and low membrane permeability (intact membrane), which is a sign of live cells. Cells in Q2 have high esterase activity and high membrane permeability, and the cells are severely damaged. In Q3 and Q4, the cells have no or low esterase activity combined with either low (Q3) or high (Q4) membrane permeability, and the cells are all dead.

3. Results

3.1. 3.1 Esterase activity and membrane integrity in *Tetraselmis suecica* incubated in dark or light

The FCM signals from dual-stained nonirradiated and UV-irradiated cells incubated in darkness displayed the same pattern with time (Fig. 2) as previously reported (Olsen et al., 2016a). The dark incubated, nonirradiated cells expressed signals indicating cells were live (Q1), but after 7 days of incubation, a small fraction of the cells (≈10%) gave signals, indicating damaged cells (Q2). The FCM dot plots from cells in UV-irradiated samples followed a different pattern. The signals displayed indicated an increasing portion of severely damaged cells (Q2) with increasing UV dose. The Q2 portion also increased as a response to dark incubation, and the percentage of severely damaged cells was ≈39%, 37%, 4%, and 1% for UV doses of 100, 200, 300, and 400 mJ/cm^2, respectively, after 7 days. During incubation, the damaged cells died (Q3 and Q4), and after 7 days of dark incubation, the percentage of dead cells was ≈4%, 33%, 94%, and 99% for UV doses of 100, 200, 300, and 400 mJ/cm^2, respectively.

The nontreated cultures responded differently to dark and light incubations (Fig. 2). As expected, the number of live cells (Q1) increased in the nontreated culture incubated in light (Fig. 3), from 9759 (± 816) cells/ml after 2 h of incubation to 284,329 (± 31,703) cells/ml after 7 days of incubation. In contrast, the live cell concentration in the nontreated cultures incubated in the dark remained unchanged almost throughout the analysis period, except for the FCM population in Q2 on day 7 (Fig. 2f).

Cells irradiated with low UV doses (100 and 200 mJ/cm^2) before light incubation, displayed FCM signals in Q1 (live) during the entire incubation period (Fig. 2). The live cell concentrations in Q1 increased from day 2 and throughout the analysis period when treated with a UV dose of 100 mJ/cm^2 (Fig. 3), ending up at a concentration of 180,000 cells/ml on day 7. Similarly, the live cell concentration increased in Q1 when treated with a UV dose of 200 mJ/cm^2 (Fig. 2), ending up with 30,000 cells/ml on day 7. The numbers of live cells in Q1 remained unchanged and only started to increase after day 4 (Fig. 3). In UV-irradiated cells incubated in the dark, a Q2 population (damaged cells) emerged first on day 7 (100 mJ/cm^2) and then on day 1 (200 mJ/cm^2) (Fig. 2f and 2b, respectively). The total numbers of cells (i.e., in all quadrants) remained unchanged when incubated in the dark for all UV-treated and nontreated cultures.

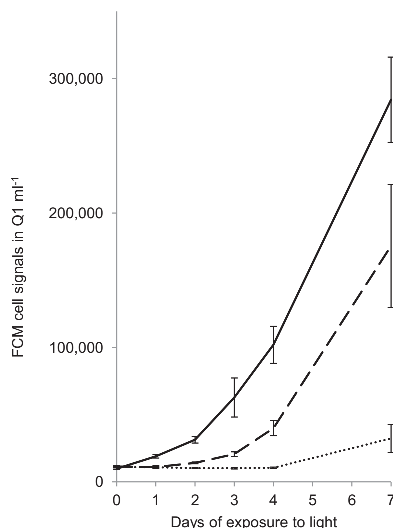

Fig. 3. FCM cell signals in Q1 (Fig. 2) after exposure to light for non-irradiated cells (__) and cultures UV treated with 100 (—) and 200 mJ/cm^2 (…). Data are means of 3 replicates and error bars indicate ± 1 SD.

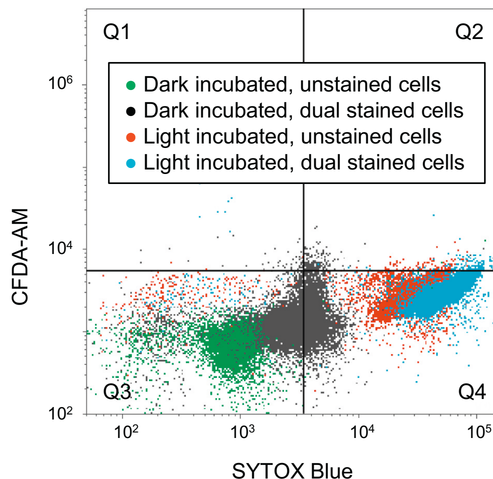

Fig. 4. FCM dot plot of Tetraselmis suecica UV treated with 400 mJ/cm^2. The figure shows FCM populations of cells incubated in 7 days in the dark or light, unstained or dual stained with SYTOX Blue and CFDA-AM. Intersections in the dot plot separate signals into 4 quadrants (Q1-4).

The most noteworthy results were observed in samples UV-irradiated with high UV doses (300 and 400 mJ/cm^2). (1) Cells exposed to light died (signals appearing in Q3 and Q4) after 2 days (dose of 300 mJ/cm^2, Fig. 2c) or 1 day (dose of 400 mJ/cm^2, Fig. 2b). Cells incubated in the dark, on the other hand, died after 7 days (dose of 300 mJ/cm^2, Fig. 2f) and 3 days (dose of 400 mJ/cm^2, Fig. 2d). (2) When exposed to light, the cells responded to increased blue fluorescence intensity during incubation (Fig. 2c) compared to that when incubated in the dark. Both stained and unstained cells displayed higher blue fluorescence intensity when incubated with light (Fig. 4). The increased blue fluorescence was first observed after 2 days (Fig. 2). (3) Damaged cells (in Q2, Fig. 2) did not resume growth, when incubated in neither light nor dark.

3.2. Chlorophyll content of *Tetraselmis suecica* incubated in dark or light

Red autofluorescence signals from nonirradiated and UV-irradiated *T. suecica* cells, incubated in light or dark, are presented as histograms (Fig. 5). The x-axis corresponds to red autofluorescence intensity, while the y-axis represents the number of events. Our results show how cells responded differently to dark and light incubation. The histograms for all dark incubated cells displayed similar fluorescence intensity during the entire analysis period (Fig. 5a, c, e, g, i). However, the cells exposed to light, responded to increased fluorescence intensity during incubation when the cells had not been treated or treated with low UV doses (100 and 200 mJ/cm^2) (Fig. 5b, d, and f). The increase in fluorescence intensity corresponded to the length of incubation; the longer the incubation, the higher is the red autofluorescence. The number of events also increased with time for these samples, which shows that the cells are dividing and growing. On the other hand, the fluorescence intensity from cells treated with high UV doses (300 and 400 mJ/cm^2) decreased during light incubation (Fig. 5h, j). The intensity decreased as a function of time after UV treatment, from relative fluorescence intensity at ~30^5 2 h after UV irradiation to ~10^4 after 7 days after treatment.

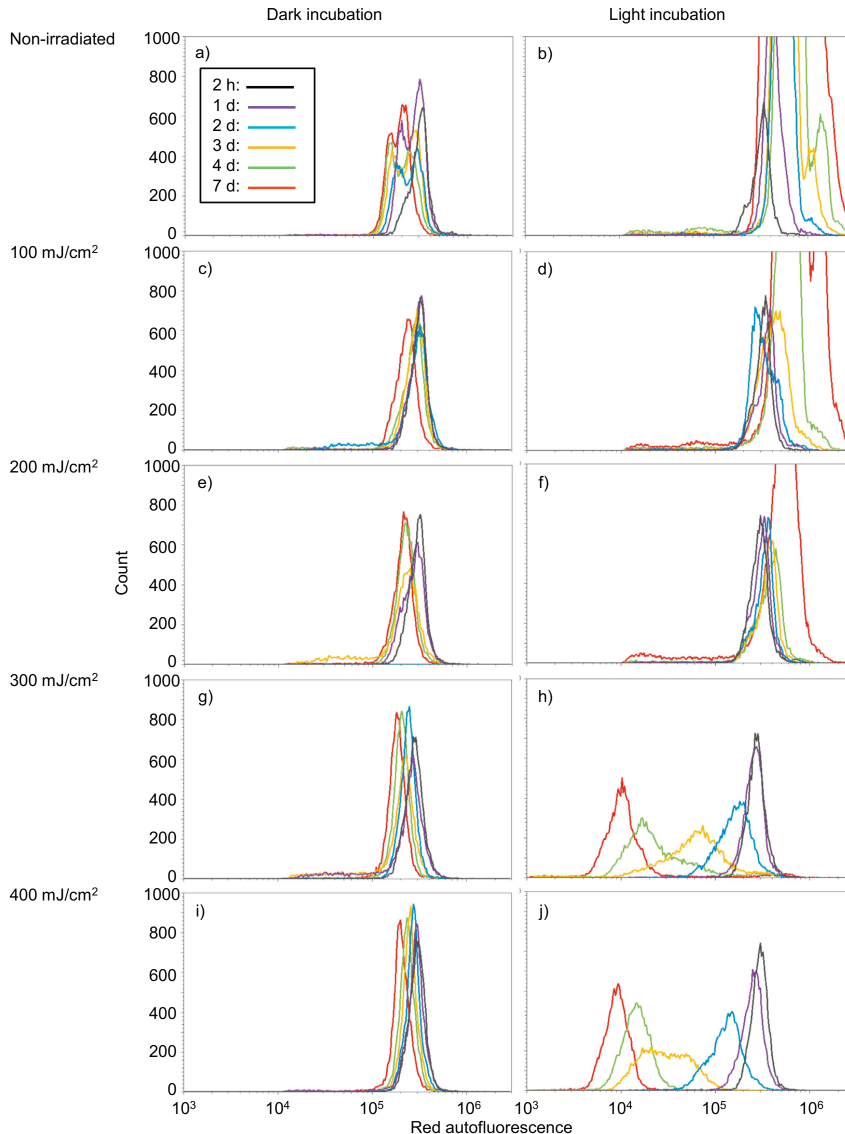

Fig. 5. Red autofluorescence.

4. Discussion

Many commercial BWTS are UV-based, with the ballast water treated at intake and at discharge (David and Gollasch, 2015; Lloyd's Register Marine, 2019). During the voyage, the ballasted water is housed in dark ballast tanks, whereas upon de-ballasting and discharge, any organism in the water will experience in situ light conditions. The current study aimed at mimicking ballast water treatment at intake (UV irradiance) before subsequent dark transport, and it inspects how such handling affects UV-treated *T. suecica* cells compared to cells that are re-incubated under light conditions.

We found that low-dose irradiation (100 and 200 mJ/cm^2) did not prevent growth after re-incubation to light and that our FCM protocol is suitable for qualitative regrowth (viability) studies when following samples over time. However, according to the G8 guidelines, treated water samples need to be analyzed live within 6 h (International Maritime Organization, 2016). We observed a longer lasting lag phase before the cells entered exponential growth phase with increasing UV dose. The UV plots did reveal vital cells in the samples during the entire incubation period (Fig. 2), and the numbers of vital cells were stable until it started to increase (Fig. 3). We therefore interpret that the delay is caused by nonviable cells, needing time to repair UV-induced damages to regain viability. When irradiated with UV doses comparable to those applied in the current study, *T. suecica* produced increasing

cellular concentrations of cyclobutane pyrimidine dimers (CPDs), a commonly UV-induced but reparable DNA damage (Weber, 2005), proportionally to increasing UV doses. After irradiation with 300 mJ/cm^2, most CPD-DNA repair occurred within 6 h of incubation and was essentially completed within 24 h (Hull et al., 2017). We argue that our results indicate that a similar photoreactivation took place in cells irradiated with low UV doses before exposure to light. It cannot be reported whether dark repair takes place in the absence of light, as *T. suecica* is a photosynthetic organism that needs light to grow and replicate. Neither light nor dark incubations promoted resumed growth of damaged cells after high UV dose (300 and 400 mJ/cm^2) treatments, demonstrating that neither membrane permeability nor vitality of damaged cells counteracted when incubated in light. We have previously demonstrated how damaged cells, when incubated in dark, can be identified as a specific FCM population and interpreted as an "early warning" of dying cells (Olsen et al., 2016a). Our current results show that this is also the case when cells are incubated in light after treatment.

From our experiment, we can conclude that MP UV irradiations using doses ≥ 300 mJ/cm^2 are lethal for *T. suecica* cells, regardless of light/dark conditions during subsequent incubation. Ballast water irradiated with such high UV doses at intake before transportation in dark ballast water tanks or treated at discharge to in situ light conditions should therefore be safe to release into the marine environment. Ballast water treated with UV doses of 100–200 mJ/cm^2 before discharge may, however, contain organisms able to grow and reproduce in the marine environment. However, it is important to keep in mind that most commercial BWTS comprise two or more treatment stages (Lloyd's Register Marine, 2019), which can enhance the inactivation at low UV doses.

Both esterase activity and chlorophyll content decreased more in *T. suecica* cells treated with high UV doses when incubated in light than in dark. Similarly, the membrane permeability was more affected when incubated in light than in dark. As the cells were treated with the same UV doses and possibly suffer from the same cellular damages, the changes in physiological characteristics are most likely caused by the combination of UV treatment and subsequent light exposure. UV light combined with high light conditions can cause formation of ROS, affecting biological molecules and causing oxidative damage to lipids, proteins, and DNA (Latowski et al., 2011; Li et al., 2009; Niyogi, 1999). For instance, high light conditions may inhibit photosynthesis by production of ROS, inactivating the photochemical reaction center of PSII or by the direct effect of light on the oxygen-evolving complex (Andersson and Aro, 2001; Aro et al., 1993; Murata et al., 2007; Powles, 1984). Photosynthetic organisms are, however, able to repair PSII damages rapidly and efficiently (Andersson and Aro, 2001; Aro et al., 1993), and photosynthetic organisms have light-absorbing protective pigments that can prevent photolytic damage to chlorophylls and the photosynthetic apparatus. Xanthophylls are effective quenchers of ROS and among the most efficient protectors against over-excitation conditions (Latowski et al., 2011). Various xanthophyll pigments such as violaxanthin, neoxanthin, antheraxanthin, and lutein are present in the chloroplasts of *T. suecica* (Sansone et al., 2017). UV treatment followed by light incubations seem, however, to have exceeded the level of stress that these protective mechanisms could handle, although the light conditions were not considered as high light conditions. Combined, these stress factors have caused nonreversible damage to cellular molecules as esterases and chlorophylls, and/or to mechanisms such as membrane permeability. *T. suecica* cells incubated in darkness were, on the other hand, not exposed to ROS and PSII damages. Less damages and a possible low metabolic activity level (Carney et al., 2011; Jochem, 1999) may also explain less effect on esterase activity, membrane integrity, and chlorophylls observed in dark-incubated *T. suecica* cells.

T. suecica cells treated with high UV doses, both dual-stained and unstained cells, exhibited higher blue fluorescence intensity after incubation in light than those when incubated under dark conditions. Cells that are exposed to stress produce or transform cellular molecules to protect, repair, and/or maintain cell integrity and metabolism, and the blue fluorescence may have been caused by accumulation of such protective substances. Emission spectra of violaxanthin or similar components do not match with our results (Sansone et al., 2017), and a previous study of the microalga *Emiliania huxleyi* showed no blue autofluorescence when under stress (Dashkova et al., 2016). On the other hand, blue fluorescence from chlorophyll degradation in higher plants has been observed (S. Hörtensteiner, 1999; Stefan Hörtensteiner and Kräutler, 2011). As chlorophyll degradation can take place as a response to biotic and abiotic stresses, we believe UV irradiation and/or high light conditions may have caused blue fluorescence from degradation products.

The current study was conducted using one model organism only, and for type approval of BWTS, *T. suecica* is suitable as a representative of the $\geq 10– < 50$ μm size category, fulfilling the biological quality of the test water (International Maritime Organization, 2008; U.S. Environmental Protection Agency, 2010). We have demonstrated that exposing *T. suecica* cells that were irradiated at low UV doses to light can impact the analysis time, as viable cells can also be detected with the FCM protocol. Moreover, at high UV doses, physiological characteristics for vitality evaluation (such as damaged/dying and dead cells) can be detected at an earlier stage when incubated in light than in dark. Ballast water will most probably contain live, damaged, and dead cells (vitality), and we have shown herein that exposing it to light has the potential to separate cells into three categories. When evaluating the performance of UV-based BWTS, these results can be useful for type approval using *T. suecica* as a test organism in the test regime. *T. suecica* is not, however, as common as diatoms, dinoflagellates, and prymnesiophytes in coastal waters, and it does not impose a health hazard to humans or sea-living organisms. Therefore, the effects of incubation regimes need to be examined on other UV-treated phytoplankton species that will be encountered in ships ballasting practice. Many marine microorganisms are heterotrophic/mixotrophic and produce energy by the oxidation of inorganic or organic compounds, independently of dark or light environmental conditions, and cellular responses to UV treatment and different incubation periods need to be confirmed for organisms less dependent on light as well.

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II

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ORIGINAL RESEARCH
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Bioorthogonal Non-canonical Amino Acid Tagging Combined With Flow Cytometry for Determination of Activity in Aquatic Microorganisms

Mathilde Lindivat[1], Aud Larsen[2,3], Ole Kristian Hess-Erga[4], Gunnar Bratbak[3] and Ingunn Alne Hoell[1]*

[1] Faculty of Engineering and Science, Institute of Safety Chemistry and Biomedical Laboratory Sciences, Western Norway University of Applied Sciences, Haugesund, Norway, [2] NORCE Environment, NORCE Norwegian Research Centre AS, Bergen, Norway, [3] Department of Biological Sciences, University of Bergen, Bergen, Norway, [4] Norwegian Institute for Water Research, Bergen, Norway

In this study, we have combined bioorthogonal non-canonical amino acid tagging (BONCAT) and flow cytometry (FCM) analysis, and we demonstrate the applicability of the method for marine prokaryotes. Enumeration of active marine bacteria was performed by combining the DNA stain SYBR Green with detection of protein production with BONCAT. After optimization of incubation condition and substrate concentration on monoculture of *Escherichia coli*, we applied and modified the method to natural marine samples. We found that between 10 and 30% of prokaryotes in natural communities were active. The method is replicable, fast, and allow high sample throughput when using FCM. We conclude that the combination of BONCAT and FCM is an alternative to current methods for quantifying active populations in aquatic environments.

Keywords: flow cytometry, protein synthesis, bioorthogonal non-canonical amino acid tagging, marine microbes, single cell activity

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****Correspondence:***
Mathilde Lindivat
mathilde.lindivat@hvl.no

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INTRODUCTION

The standard approach for assessing heterotrophic prokaryotic production in natural ecosystems is to measure incorporation of radioactive labeled leucine or thymidine (Bell, 1993; Kirchman, 1993). These methods report activity in terms of substrate uptake and/or incorporation or number of new cells produced per unit of time and volume, respectively. They have indeed been instrumental for establishing the role of bacteria in natural food webs. As bulk measurements, the values relate to the prokaryotic community as a whole. Bacterioplankton communities in natural aquatic ecosystems are, however, generally diverse and made up of many different populations. Each of them grows at their own pace depending on their physiological abilities and how well they are adapted and able to cope with the environment in which they live (Alonso-Sáez and Gasol, 2007; Del Giorgio and Gasol, 2008). Knowing how growth and activity varies and how they are distributed allow for a better understanding of the heterogeneity of microbial communities and how they function in the ecosystem.

A number of different methods have been used to investigate the vitality or fraction of active cells in prokaryote communities. These include microautoradiography to visualize incorporation of radioactive labeled substrates (Cottrell and Kirchman, 2004; Sintes and Herndl, 2006), different fluorescent stains that assesses cell membrane integrity or intracellular

enzyme activity (Manini and Danovaro, 2006; Del Giorgio and Gasol, 2008), and use of tetrazolium redox dyes like CTC (5-cyano-2,3,-ditolyl tetrazolium chloride) that are reduced to fluorescent formazan in respiring cells (Rodriguez et al., 1992; Smith Erik, 1998). However, some of these methods, like the CTC and Redox assays, have shown biased results when trying to measure cell activity rates (Ullrich et al., 1999; Servais et al., 2001; Hammes et al., 2011; Netuschil et al., 2014; Emerson et al., 2017; Hatzenpichler et al., 2020). More recently, incorporation of amino acid analogues has been used to show protein synthesis in natural prokaryote communities by a click chemistry protocol termed bioorthogonal noncanonical amino acid tagging (BONCAT; Hatzenpichler et al., 2014). In short, microbial assemblages are incubated with azide- or alkyne-bearing methionine analogs [i.e., L-azidohomoalanine (AHA) or L-homopropargylglycine (HPG)]. Analogues incorporated into newly synthetized proteins are then made fluorescent by conjugation to alkyne- or azide-labeled fluorophores in a copper (I) catalyzed azide-alkyne cycloaddition chemistry reaction (a click reaction). Cells with active protein synthesis are hence made visible by standard epifluorescence microscopy (Hatzenpichler et al., 2014; Hatzenpichler and Orphan, 2015).

Bioorthogonal noncanonical amino acid tagging has successfully been used to measure activity in approximately 30 members of cultured and uncultured prokaryote phyla (Hatzenpichler et al., 2020). This suggests that BONCAT can be broadly applied to taxonomically different microorganisms. The applicability of the method has also been tested on prokaryote communities in freshwater and freshwater sediments, as well as in different marine systems and deep sea sediments (Hatzenpichler et al., 2014, 2016; Samo et al., 2014; Hatzenpichler and Orphan, 2015; Leizeaga et al., 2017). BONCAT has, in addition, been used to follow protein synthesis during viral infections of both bacteria and marine phytoplankton (i.e., *Escherichia coli*/T7, *Synechococcus* sp. WH8101/Syn1, and *Emiliania huxleyi*/EhV207; Pasulka et al., 2018) and to study microbial activity in soil communities (Couradeau et al., 2019) or the response of bathypelagic prokaryotes to starvation (Sebastián et al., 2019).

Flow cytometry (FCM) has become a widely used method for quantifying and analyzing aquatic microorganism, including phytoplankton, heterotrophic flagellates, bacteria, and viruses (Marie et al., 1997, 1999; Manti et al., 2012). In comparison to microscopy, FCM is more reliable and reproducible because there is less bias by operator experience and it is faster with lower running cost allowing for a much higher sample throughput. In addition, it may allow for fluorescence-activated cell sorting (FACS) and further interrogation of specific subpopulations of interest. Combining FCM and BONCAT should thus be advantageous and provide a robust method for microbial activity analysis with FCM. The usefulness has indeed been demonstrated by sorting of BONCAT labeled cells (BONCAT-FACS) for identification of active cells in complex samples (Hatzenpichler et al., 2016) and soil microbial communities (Couradeau et al., 2019), but not for measuring activity in marine samples. The possibility to use different dyes for BONCAT detection, as presented in **Figure 1**, allows the application to different FCM setups and for different purposes such as activity monitoring.

The main aim of this work was to establish a BONCAT protocol for FCM analysis of prokaryotic activity to be used alongside a standard FCM protocol for enumeration of bacteria. Secondly the application of BONCAT-FCM to marine microorganisms was evaluated. The protocol was developed and optimized using pure bacterial cultures and mixed marine bacterial communities, and we used different combinations of stains for total count and protein production to allow analysis for instruments with different laser combinations.

Abbreviations: AF, Alexa fluor; AHA, L-azidohomoalanine; BONCAT, Bioorthogonal non-canonical amino acid tagging; CTC, 5-cyano-2,3,-ditolyl tetrazolium chloride; FACS, Fluorescence-activated cell sorting; FCM, Flow cytometry method; FISH, Fluorescence *in situ* hybridization; HPG, L-homopropargylglycine; THPTA, Tris [(1-hydroxypropyl-1*H*-1,2,3-triazol-4-yl)methyl]amine.

MATERIALS AND METHODS

Incubation of Model Species and Natural Marine Bacterioplankton

Escherichia coli (ATCC 25922) was used as model strains in this study. *E. coli* was cultured in M9 minimal media [0.5 g NaCl, 2 g glucose, 1 g NH_4Cl, 12.8 g Na_2HPO_4•$7H_2O$, 3 g KH_2PO_4, 0.492 g $MgSO_4$•$7H_2O$, 0.11 g $CaCl_2$, and 0.1 g Thiamine pr. 1 L milliQ water (all chemicals purchased from Merck, Germany)], and incubated at 37°C. Natural marine microbial communities from surface water (<5 m) were collected in Karmsundet (Haugesund, Norway) and Puddefjorden (Bergen, Norway) between June 2017 and August 2019. Sea water was prefiltered at 100 μm to avoid instrument clogging. Incubations were set up directly after sampling as show in experimental set up in **Figure 2**.

Stock solutions of 100 mM L-homopropargylglycine (HPG) pH 7.0 (Click Chemistry Tools, USA) were prepared in pure DMSO, sterile 0.2 μm-filtered and kept at 4°C. HPG diluted in milliQ water was added to pure cultures when they reached exponential growth phase (~0.3–0.4 OD) at a final concentration of 20 nM, 50 nM, 1 μM, and 5 μM in triplicate samples. Negative control samples were prepared without HPG. Twenty five micromolar of chloramphenicol (Sigma Aldrich, USA) was added to stop protein production in control samples with HPG (Chloramphenicol control). *E. coli* and marine samples were subsequently incubated in the dark from 1–24 h and 1–48 h, respectively. After incubation, samples were fixed with 0.2 μm-filtered formaldehyde (Sigma Aldrich, USA) to a final concentration of 3% before storing at −20°C prior to the click chemistry reaction if not analyzed directly.

Click Chemistry Protocol Validation

A protocol adapted from the Hatzenpichler group and the Samo group (Samo et al., 2014; Hatzenpichler and Orphan, 2015) was set up with Alexa Fluor® azides 488, 405, and 647 (Thermofisher Scientific, USA). After incubation with HPG and fixation, samples were centrifuged 5 min at 16,000 × *g* (Thermo Scientific, Multifuge 3SR+). Cells were resuspended in 1 ml 0.2 μm-filtered 1x

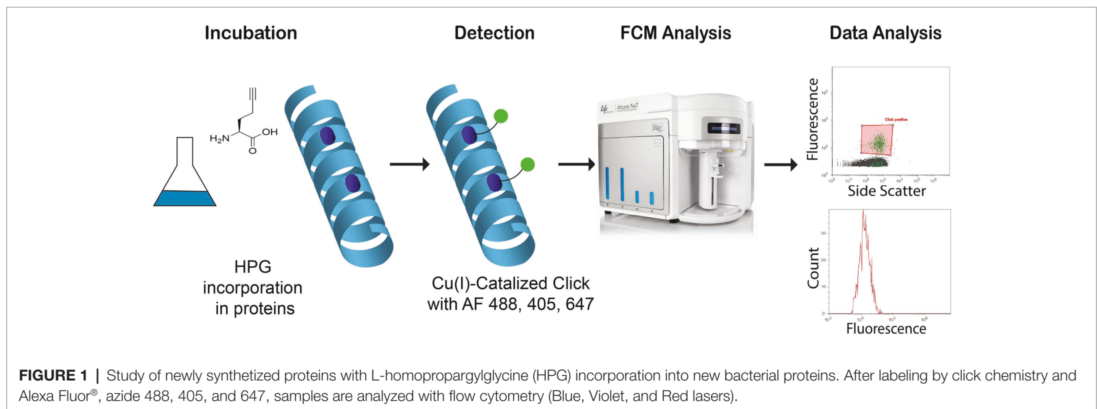

FIGURE 1 | Study of newly synthetized proteins with L-homopropargylglycine (HPG) incorporation into new bacterial proteins. After labeling by click chemistry and Alexa Fluor®, azide 488, 405, and 647, samples are analyzed with flow cytometry (Blue, Violet, and Red lasers).

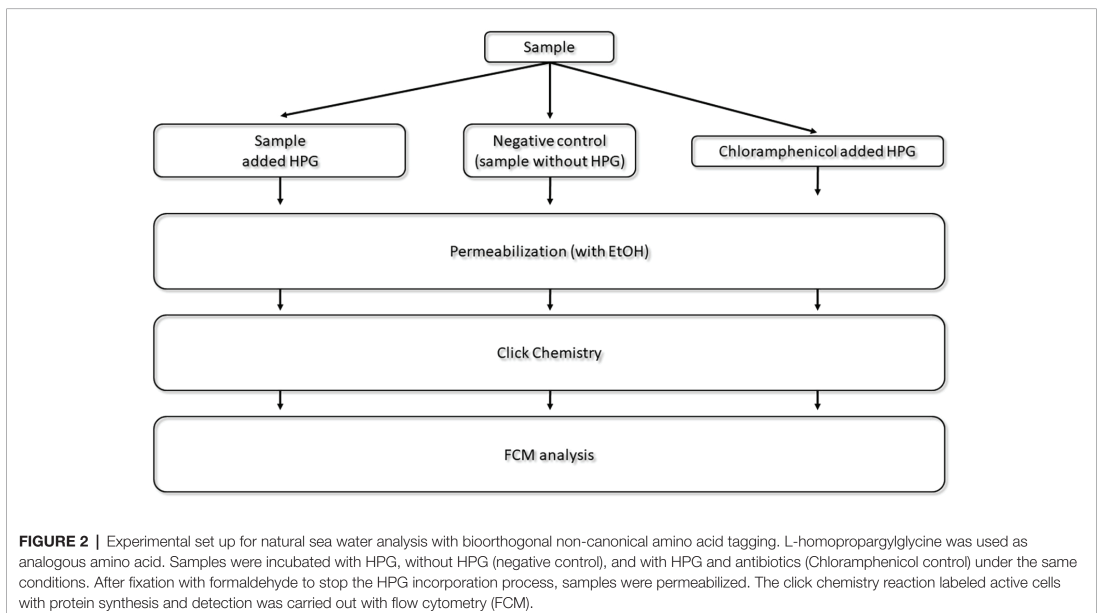

FIGURE 2 | Experimental set up for natural sea water analysis with bioorthogonal non-canonical amino acid tagging. L-homopropargylglycine was used as analogous amino acid. Samples were incubated with HPG, without HPG (negative control), and with HPG and antibiotics (Chloramphenicol control) under the same conditions. After fixation with formaldehyde to stop the HPG incorporation process, samples were permeabilized. The click chemistry reaction labeled active cells with protein synthesis and detection was carried out with flow cytometry (FCM).

phosphate-buffered saline (PBS), pelleted by centrifugation, and then resuspended and treated using 1 ml 50% ethanol followed by 3 min incubation at room temperature (RT). Ethanol was removed after centrifugation 5 min at 16,000 × *g*. Next, incubations with ethanol 80 and 96% were carried out identically. Finally, cells were washed and suspended in 1 ml 1x PBS.

The following solutions were prepared before starting the click chemistry reaction: (1) copper sulfate ($CuSO_4$•5 H_2O; Jena Bioscience, Germany) was diluted in MilliQ water to a final concentration of 20 mM and stored at 4°C, (2) stock solutions of 50 mM Tris [(1-hydroxypropyl-1*H*-1,2,3-triazol-4-yl)methyl]amine (THPTA; Click Chemistry Tools, USA) was prepared in MilliQ water and stored in aliquots at −20°C, and (3) fresh solution of 100 mM sodium ascorbate (Sigma Aldrich, USA) in 1x PBS and 100 mM aminoguanidine hydrochloride (Sigma Aldrich, USA) in 1x PBS.

Click chemistry reaction was performed in the dark starting with a 3 min dark incubation of the pre-mix solution containing ~100 μM $CuSO_4$, 500 μM THPTA (ratio of 5:1 of $CuSO_4$), and 5 μM azide dye [Alexa Fluor® 488 (AF488), Alexa Fluor® 405 (AF405) or Alexa Fluor® 647 (AF647)]. Sodium ascorbate (5 mM final concentration) and aminoguanidine hydrochloride (5 mM final concentration) were added directly to each sample, followed by 17.5 μl of pre-mix. All reactions were carried out in eppendorf tubes, in a final volume of 1.15 ml.

Samples were gently mixed by turning the tube, before incubation for 30 min in the dark. After incubation, samples were centrifuged at 16,000 × *g* for 5 min, washed two times with 1x PBS, and finally suspended in sterile 1x PBS or 1x TE-buffer.

Double Staining Procedure

Total bacterial numbers were determined by flow cytometry after DNA staining using either LDS 751 or SYBR Green. Samples with azide AF488 labeled proteins were stained with 10 μl of 20 μg/ml LDS 751 stock solution (Invitrogen, USA), followed by incubation for 10–15 min in the dark at RT. Samples with AF405 azide and AF647 azide labeled proteins were stained with 10 μl of 100x SYBR Green (Invitrogen, USA) for 10–15 min in the dark at RT, following an adaptation of Marie et al. (1999) protocol for marine samples. No washing steps were performed after the double staining procedure.

Flow Cytometry Analysis

Flow cytometry analysis was performed with an Attune NxT Acoustic Focusing Cytometer (Thermofischer scientific, USA) with a Violet laser 405 nm (50 mW), a Blue laser 488 nm (50 mW), and a Red laser 638 nm (100 mW). Attune Performance tracking beads (2.4 and 3.2 μm; Thermofischer, USA) were used for instrument calibration. The following detectors were used for fluorescence detection according to dyes excitation and emission wavelength (**Table 1**): BL1 (530/30) for SYBR Green and Alexa Fluor® 488 azide (AF488), VL1 (440/50) for Alexa Fluor® 405 azide (AF405), RL1 (670/14) for Alexa Fluor® 647 azide (AF647), and LDS 751. Trigger was set at 2000 on Side Scatter SSC-H (for LDS 751) or on BL1 (for SYBR Green). Voltages were optimized for each detector. SYBR Green was used in combination with AF405 azide or AF647 azide, whereas LDS 751 was used with AF488 azide. For seawater analysis, size-standardized beads ranging from 0.5 to 2 μm (Thermofisher Scientific, USA) were used to determine bacterial and virus like particles populations. Between 200 and 2,000 cells were analyzed at a flow rate of 25 $\mu l \cdot min^{-1}$. Counts were performed on three biological replicates and each of samples were analyzed three times. Counts were also performed on higher cell numbers (20,000 and 200,000) and showed similar results than lower cell numbers analysis (200, 2,000).

Microscopy Analysis

After the click chemistry reaction, samples were filtered onto black polycarbonate 0.2 μm filters (Whatman, UK), stained with SYBR Green, and rinsed with sterile 1x PBS. Samples were counted in a Nikon Optiphot-2 microscope (Nikon Instruments, Japan) with Hg lamp C-SHGI (Nikon Instruments, Japan). Images were obtained with NIS-Elements 2.20 (Nikon Instruments). The fluorescence filters used were B-2A (Ex450-490) for AF488 azide and SYBR Green, and G2-A (Ex510-560) for LDS 751.

TABLE 1 | Overview of excitation and emission wavelength for DNA and click dyes used in the study.

Dyes	Excitation (nm)	Emission (nm)
SYBR Green	497	520
LDS 751	543	712
AF405 azide	401	421
AF488 azide	490	525
AF647 azide	650	665

RESULTS AND DISCUSSION

Optimization and Application of BONCAT to FCM

Incubation conditions for the proposed method to measure activity in single bacterial cells using flow cytometry were optimized with pure *E. coli* cultures grown on minimal media M9 to deplete methionine stocks (Dieck et al., 2012) and increase L-homopropargylglycine (HPG) incorporation. BONCAT incorporation was tested for a range of HPG concentrations from 20 to 5 μM with LDS 751/AF488 azide dyes. The highest level of incorporation was observed after 1–3 h incubation time using 5 μM HPG (91% of active cells; **Figure 3**). Low HPG concentrations (20 and 50 nM) were not suitable for long time incubations as numbers of positive cells was low and standard deviations between three replicates were high. Depletion of HPG over time due to the increase in cell population during exponential growth will lead to a reduction in the number of detected active cells. We suggest to use high HPG concentration (>15 μM) for monoculture incubation. As reported previously (Hatzenpichler and Orphan, 2015; Hatzenpichler et al., 2016; Leizeaga et al., 2017; Pasulka et al., 2018), we did not find that high concentrations (over 1 μM) of HPG affected cell growth positively nor negatively. Negative controls without HPG showed that false positive cells varied between 0 and 13% during incubation and that a higher content of false positive click cells were observed after short (1–2 h) compared to long incubation times (up to 24 h). However, this number was influenced by the use of the LDS751/AF488 azide. Whether LDS751 was suitable for separating the bacterial population from the background noise can be questioned as we observed a much lower false positive rate for SYBR Green/AF647 azide (0.01–0.1%). Chloramphenicol controls were included in the experiments to confirm HPG incorporation only in vital cells (Samo et al., 2014). HPG incorporation stopped during the first 2 h after addition of chloramphenicol and then stabilized, verifying stop in protein production (**Figure 4**).

The click reaction in liquid was a critical step of the protocol. The oxidation state of Cu(I) has to be maintained during the conjugation of the analogous amino acid (Presolski et al., 2011); we therefore carried out the reaction in 1,000 μl volume. Cell pelleting and supernatant removal for washing steps and ethanol treatment caused around 11 ± 2% cell loss for *E. coli* samples and need to be done with care to limit cell loss. A greater cell loss can be expected with natural communities due to their relative small sizes. Couradeau et al. (2019) proposed an alternative method where cells were filtered, clicked, and then vortexed to resuspend the cells from the filter. The amount of

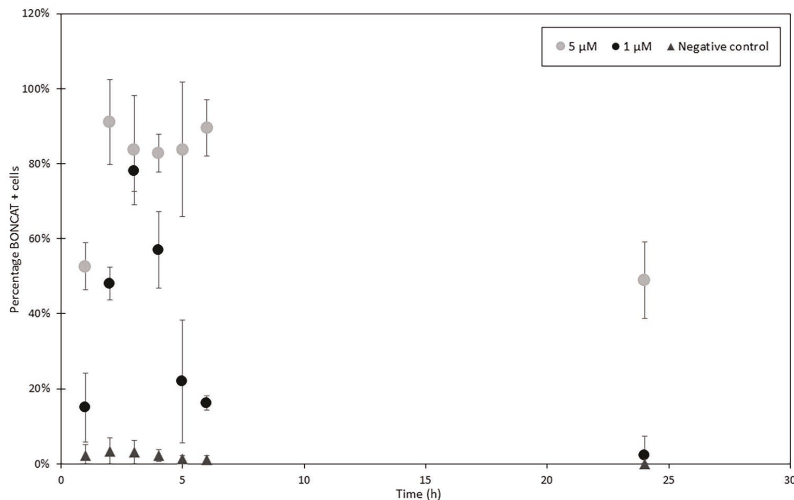

FIGURE 3 | HPG incorporation in *Escherichia coli* over time with 1 µM (black) and 5 µM (gray) and control (dark gray). HPG samples were collected every hour and then at 24 h after the incubation start and analyzed with FCM and LDS 751/AF488 azide. Percentages of positive cells were determined comparing to total cell enumeration (2,000 cells analyzed per samples in triplicates). Activity increased in the first 3–4 h to decrease in the following hours. After, new produced cells do not incorporate HPG combined with a continuous increase in cell concentration leading to a diminution of detected activity.

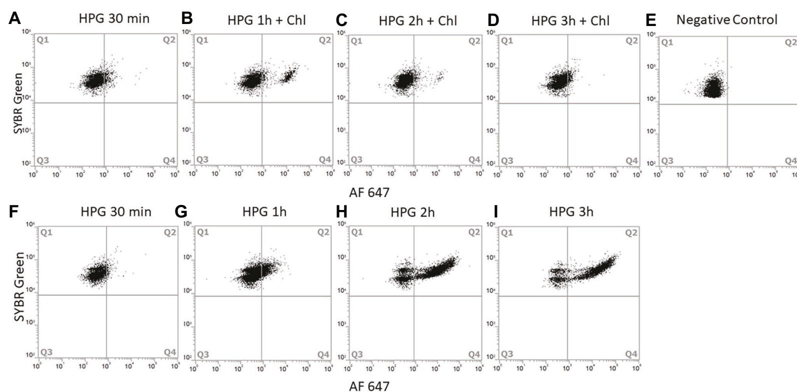

FIGURE 4 | Chloramphenicol action on protein production of *E. coli*. Chloramphenicol was added after 30 min of incubation with HPG and samples were collected every hour. Cells were analyzed with SYBR Green/AF647 azide and FCM (10,000 cells analyzed). Dot plot of *E. coli* cells incubated for: 30 min with HPG **(A)**, 1 h with HPG and 30 min with chloramphenicol **(B)**, 2 h with HPG and 1.5 h with chloramphenicol **(C)**, 3 h with HPG and 2.5 h with chloramphenicol **(D)** Negative control without HPG **(E)**. Respective positive control incubation of *E. coli* with HPG for 30 min **(F)**, 1 h **(G)**, 2 h **(H)**, and 3 h **(I)**. Q1 contain negative BONCAT cells; Q2 contain positive BONCAT cells (active). Positive BONCAT cells (=active cells) increased to 6 ± 0.7% during the first 30 min of incubation with HGP **(A)**. After adding chloramphenicol, activity stabilized to 8.8 ± 1.7% **(B)** and started to decrease at 2 h **(D)** whereas the positive control increased from 3 ± 0.4% **(F)** to 18.6 ± 2% **(G)**, 71.2 ± 0.3% **(H)** and 79.6 ± 0.1% **(I)**.

cells resuspended varied between samples causing biased results. None of the methods managed to capture 100% of the cells.

The two DNA stains tested, LDS751 and SYBR Green, gave comparable numbers for *E. coli* cultures (similarity of 96 ± 5.1%; **Figures 5A,B**). For discrimination between bacteria and background noise, as well as between various microbial populations in marine environmental samples, LDS751 (**Figure 5C**) performed poorer than the well-established marine microbes stain SYBR Green (Noble and Fuhrman, 1998; Marie et al., 1999). As described by Marie et al. (1999), various virus like populations

(V1, V2, and V3) were observed for marine samples in addition to bacteria (**Figure 5D**). When comparing active vs. total *E. coli* cell numbers analyzed with FCM and microscopy counts, the results agreed to a large extent (**Table 2**). Nevertheless, when red emitting dyes like AF647 were used, FCM was more sensitive than epifluorescent microscopy as cells with low fluorescence that were detected by FCM were difficult to see in the microscope (data not shown). Since the performance with SYBR Green was better than LDS for marine bacteria, we chose the SYBR Green counterstaining protocol for our final BONCAT procedure. LDS 751 in combination with Alexa Fluor® 488 azide (AF488) dye was used for *E. coli* only to optimize HPG concentration. The result of the combined staining is presented in **Figure 6** where the number of total active bacterial numbers, as determined by FCM, derives from the dot plots (**Figures 6A,C,E**) and the corresponding histograms provide the distribution of active bacterial population (**Figures 6B,D,F**). These plots show that AF488 azide and AF647 azide gave cells with highest fluorescence intensities that also separated well from background noise (**Figures 6A,E**). A difference of laser power (100 mW for red laser and 50 mW for violet laser) could explain the variation of separation observed for AF647 azide and AF405 azide. We agree with Hatzenpichler and Orphan (2015) that the optimum concentration for azide dyes in click reactions is 5 μM. **Figure 7** shows the BONCAT/DNA staining for all used dyes and how quadrants gating allows to separate active from non-active cells and the background whereas **Figures 6B,D,F**, show how the incorporation of HPG follow a normal distribution, as expected for cells in exponential growth phase, indicating that the cells are highly active and incorporate similar amount of HPG.

BONCAT, Flow Cytometry, and Activity of Marine Microbial Communities

The adjusted BONCAT methodology applied on marine environmental samples produced results which show that FCM distinguishes active cells from non-active cells and background

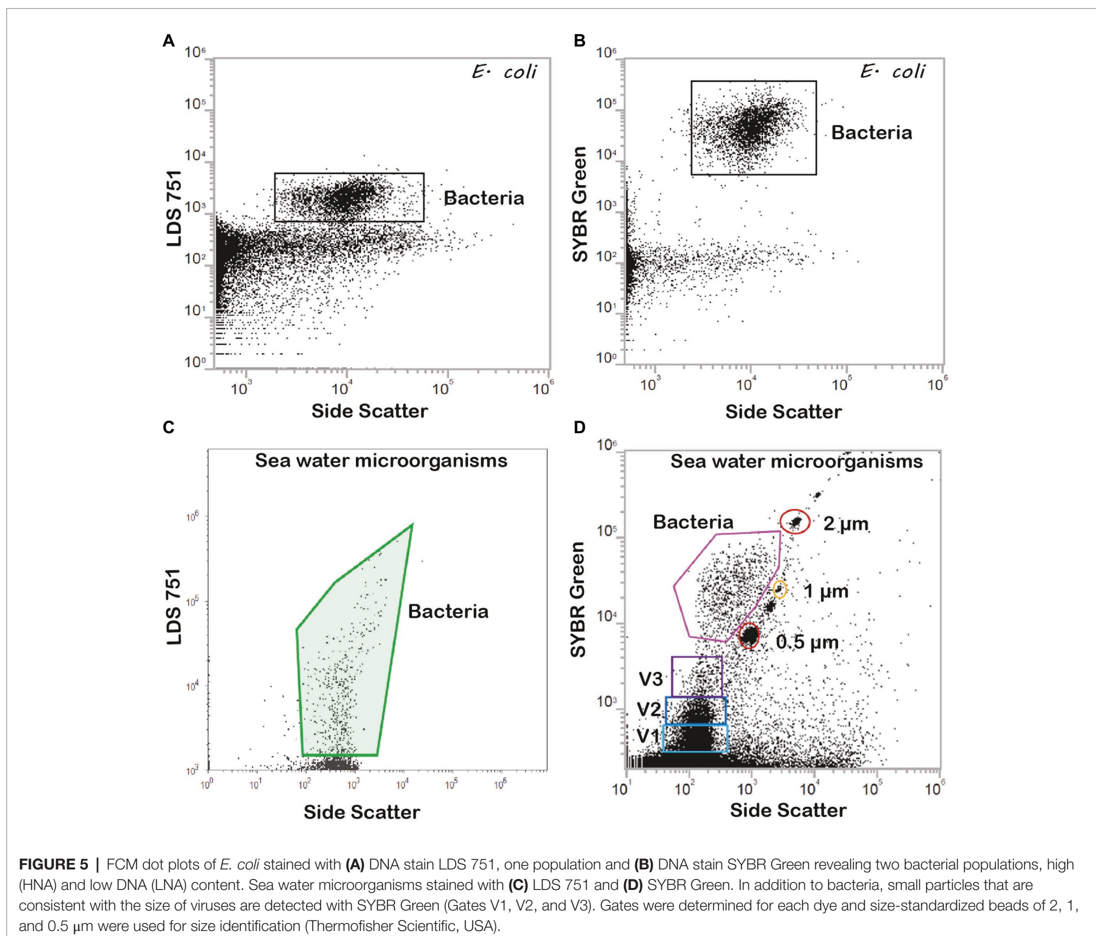

FIGURE 5 | FCM dot plots of *E. coli* stained with **(A)** DNA stain LDS 751, one population and **(B)** DNA stain SYBR Green revealing two bacterial populations, high (HNA) and low DNA (LNA) content. Sea water microorganisms stained with **(C)** LDS 751 and **(D)** SYBR Green. In addition to bacteria, small particles that are consistent with the size of viruses are detected with SYBR Green (Gates V1, V2, and V3). Gates were determined for each dye and size-standardized beads of 2, 1, and 0.5 μm were used for size identification (Thermofisher Scientific, USA).

TABLE 2 | Comparison microscopy-flow cytometry (FCM).

Incubation time (h)	Microscopy		FCM	
	Total cells/ml	% Positive	Total cells/ml	% Positive
1	1.20E+07	34 ± 4	1.71E+07	42 ± 1
2	1.07E+07	83 ± 11	8.74E+06	96 ± 1
3	1.40E+07	63 ± 4	1.62E+07	74 ± 8
4	2.00E+07	34 ± 26	1.70E+07	61 ± 3

Epifluorescence microscopy and FCM analysis of Escherichia coli incorporation of L-homopropargylglycine labeled with AF488 azide and DNA stained with LDS 751.

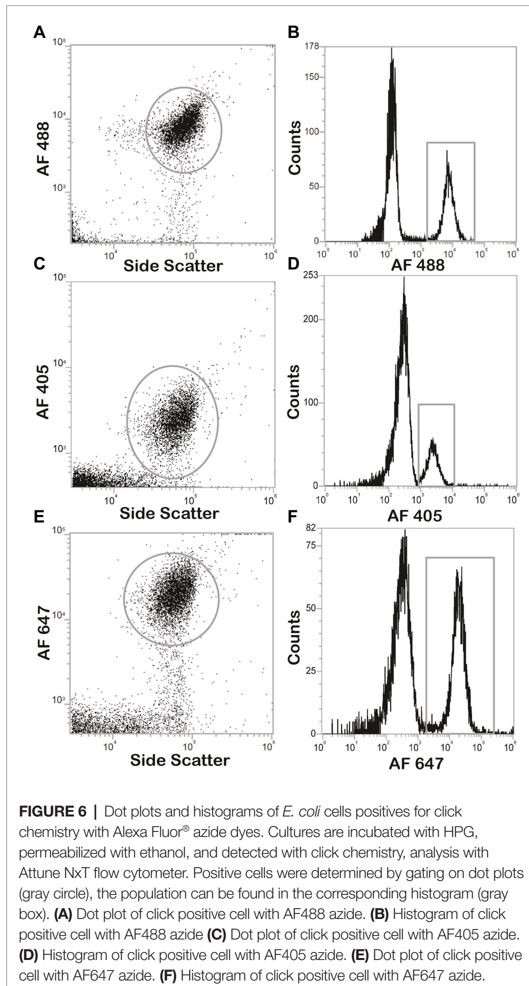

FIGURE 6 | Dot plots and histograms of *E. coli* cells positives for click chemistry with Alexa Fluor® azide dyes. Cultures are incubated with HPG, permeabilized with ethanol, and detected with click chemistry, analysis with Attune NxT flow cytometer. Positive cells were determined by gating on dot plots (gray circle), the population can be found in the corresponding histogram (gray box). **(A)** Dot plot of click positive cell with AF488 azide. **(B)** Histogram of click positive cell with AF488 azide **(C)** Dot plot of click positive cell with AF405 azide. **(D)** Histogram of click positive cell with AF405 azide. **(E)** Dot plot of click positive cell with AF647 azide. **(F)** Histogram of click positive cell with AF647 azide.

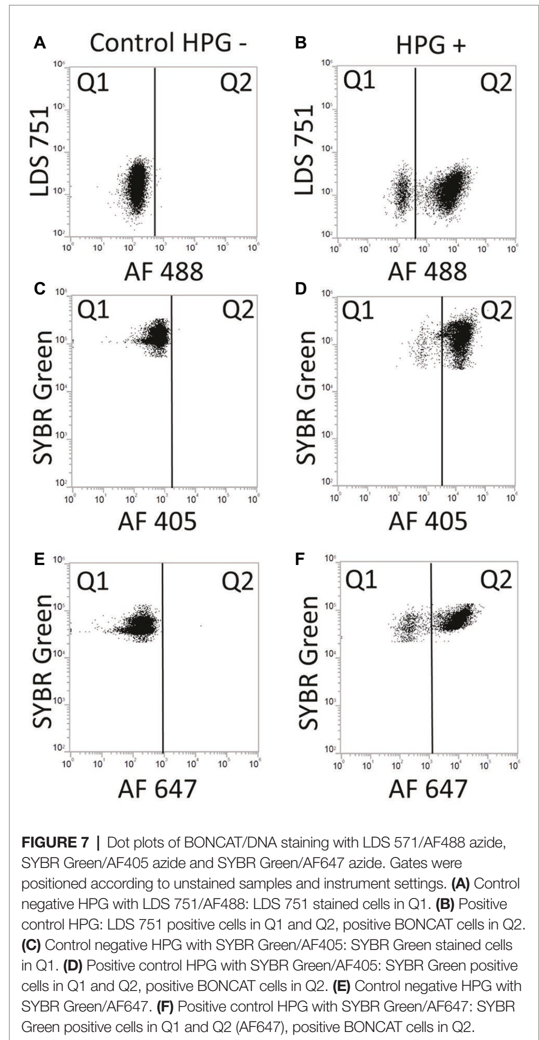

FIGURE 7 | Dot plots of BONCAT/DNA staining with LDS 571/AF488 azide, SYBR Green/AF405 azide and SYBR Green/AF647 azide. Gates were positioned according to unstained samples and instrument settings. **(A)** Control negative HPG with LDS 751/AF488: LDS 751 stained cells in Q1. **(B)** Positive control HPG: LDS 751 positive cells in Q1 and Q2, positive BONCAT cells in Q2. **(C)** Control negative HPG with SYBR Green/AF405: SYBR Green stained cells in Q1. **(D)** Positive control HPG with SYBR Green/AF405: SYBR Green positive cells in Q1 and Q2, positive BONCAT cells in Q2. **(E)** Control negative HPG with SYBR Green/AF647. **(F)** Positive control HPG with SYBR Green/AF647: SYBR Green positive cells in Q1 and Q2 (AF647), positive BONCAT cells in Q2.

particles (**Figure 8**). Incubations of marine samples with HPG were carried out over 48 h and analyzed with SYBR Green-AF647 azide. We recommend AF647 azide dye for BONCAT to avoid noise contamination from the sample (observed with AF405). Prokaryotic abundances ranged from 1.6×10^5 to 1.4×10^6 cells/ml, which are common concentrations in Norwegian coastal waters (**Table 3**; Eilers et al., 2000; Larsen et al., 2004; Brakstad and Lødeng, 2005; Reinthaler et al., 2005). More importantly, activity levels (between 6 and 39% active cells) measured using BONCAT were in accordance with levels measured using other methods (esterase activity, CTC, microautoradiography etc.) which often report that 10–60% of the cells are active (Del Giorgio and Gasol, 2008). Prokaryotes growth rates and biomass production distribution characterize communities and how much they contribute to element cycling, energy flow, and other natural processes such as viral lysis (Kirchman, 2016). BONCAT combined with FCM allows identification of the active part of a community at a single cell level. This enables the exploration

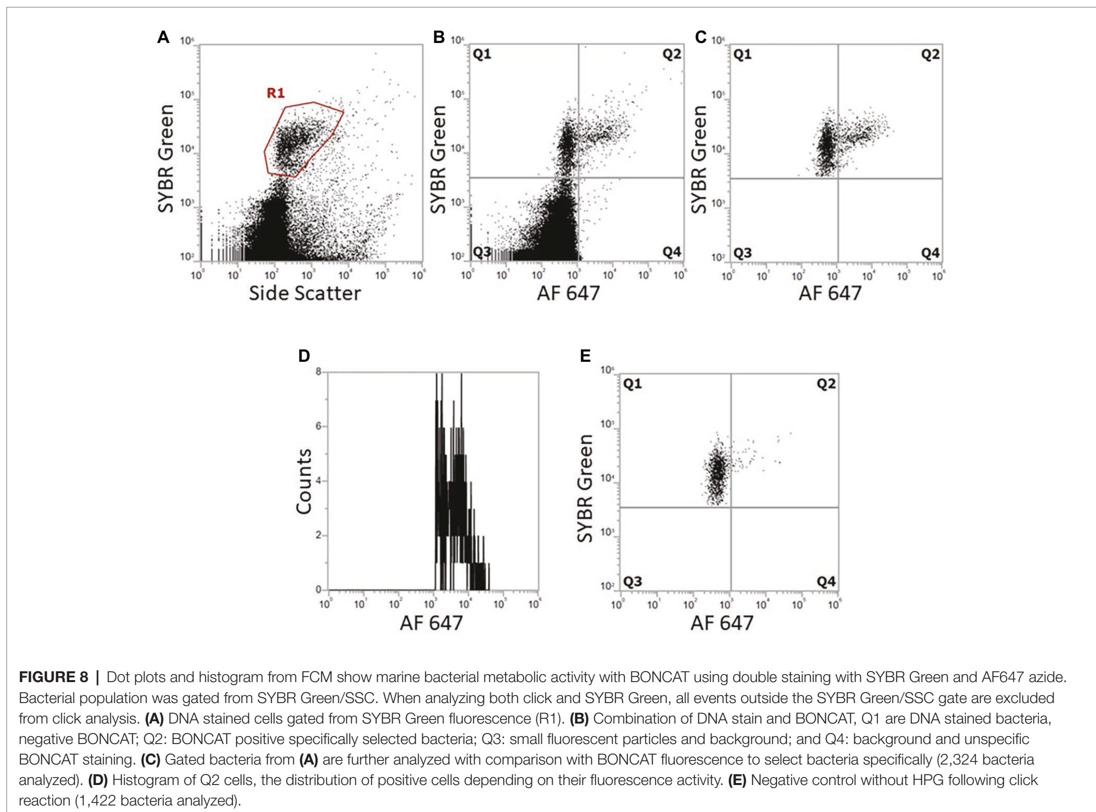

FIGURE 8 | Dot plots and histogram from FCM show marine bacterial metabolic activity with BONCAT using double staining with SYBR Green and AF647 azide. Bacterial population was gated from SYBR Green/SSC. When analyzing both click and SYBR Green, all events outside the SYBR Green/SSC gate are excluded from click analysis. **(A)** DNA stained cells gated from SYBR Green fluorescence (R1). **(B)** Combination of DNA stain and BONCAT, Q1 are DNA stained bacteria, negative BONCAT; Q2: BONCAT positive specifically selected bacteria; Q3: small fluorescent particles and background; and Q4: background and unspecific BONCAT staining. **(C)** Gated bacteria from **(A)** are further analyzed with comparison with BONCAT fluorescence to select bacteria specifically (2,324 bacteria analyzed). **(D)** Histogram of Q2 cells, the distribution of positive cells depending on their fluorescence activity. **(E)** Negative control without HPG following click reaction (1,422 bacteria analyzed).

TABLE 3 | Detection of marine bacteria activity with SYBR Green/AF647 azide after incubation with HPG.

Date	Location	Incubation time (h)	Total bacteria/ml	Number of cell analyzed	Positive active cells (%)
22.10.2018	Haugesund	6	4.20E + 05	222	6.42 ± 5.60
22.10.2018	Haugesund	6	4.36E + 05	267	10.08 ± 1.49
22.10.2018	Haugesund	6	5.20E + 05	204	8.40 ± 12.40
22.10.2018	Haugesund	6	4.08E + 05	281	13.60 ± 12.40
27.11.2018	Haugesund	6	1.29E + 06	232	39.90 ± 1.56
27.11.2018	Haugesund	6	1.43E + 06	302	30.19 ± 3.67
05.02.2017	Haugesund	2	1.46E + 05	384	30.45 ± 0.11
05.02.2018	Haugesund	5	1.57E + 05	270	30.75 ± 4.64
05.02.2019	Haugesund	24	1.84E + 05	520	23.2 ± 2.4
05.02.2019	Haugesund	8	1.69E + 05	460	26.75 ± 0.8
06.09.2019	Puddefjord	1	1.80E + 05	2,324	25.1 ± 1.55

Samples were incubated in replicates. Different incubation time are represented here. In general, 6 h incubation was selected. Total bacterial number was evaluated for each sample.

of activity at a higher resolution and thus facilitate further analysis to go deeper into their role in the environment.

One challenge when trying to describe microbial activity by FCM is that the fluorescence level of dormant or low activity cells is so low that they are masked by background noise. Our results show how the resulting underestimation of actual bacterial activity can be avoided by double staining, which enables differentiation of particles from microbes, improves the flow cytometric signal:noise ratio, and allows the determination of percentage of active cells in a population.

The combination of DNA stains and activity stains allows for a two-step detection: (1) identification of microbial cells and (2) detection of active cells, and thus extract them from background noise as demonstrated in **Figure 8**. Hatzenpichler et al. (2016) did not use counter staining for cells sorting. For our analysis, side scatter (SSC) was selected as the second parameter to create plots for each dye. Couradeau et al. (2019) used forward scatter (FSC) in their study. Both parameters are representative of cell characteristics with FSC for cell size and SSC for cell granularity or complexity. However, SSC is more commonly used for small particle analysis (such as microbes) as the signal is detected using a more suitable photomultiplier tube (PMT) than FCS (Shapiro, 2003). SSC is also more consistent between instruments compared to FSC where variations can be observed (Gasol and Del Giorgio, 2000).

Several concentration of HPG were evaluated, and contrary to Samo et al. (2014) who used low HPG concentrations (20 nM), we found that also for marine prokaryotes, higher HPG concentrations (1 μM) increase the levels of detection for low fluorescent cells. Lower HPG concentration 20 and 50 nM produced very poor results with FCM. The optimal time for incubation with HPG can vary depending on the type of samples. For slow growing organisms from deep sea environment (Hatzenpichler and Orphan, 2015; Hatzenpichler et al., 2016), a long incubation time is necessary (>100 days). For surface water microorganisms, Leizeaga et al. (2017) proposed an incubation time of 1 or 4 h to evaluate metabolic activity in marine microbial community whereas Samo et al. (2014) selected 1 h incubation. Here, we analyzed metabolic activity over 48 h with regular sampling between 1 h and 8 h, as well as at 24 h and 48 h. Longer incubations may result in community changes that can influence the overall and initial activity. Our study showed incorporation of HPG during the first hours (1–3 h), and percentage of active cells remained stable over time (**Figure 9**). Several features may explain the stabilization of the fraction of labeled cells (i.e., cells above some detection threshold) including growth rate distribution, cell size (protein content) distribution, depletion of substrate, starvation or dormancy state, cellular protein turnover, and initial surplus (luxury) uptake of substrate (Del Giorgio and Gasol, 2008). Thorough studies with variation of multiple factors are necessary to interpret and understand the results obtained with BONCAT analysis when applied to natural microbial communities.

We have demonstrated the possible use of FCM for BONCAT analysis. Our results show that the combination BONCAT-DNA staining with FCM represent a reliable protocol for analysis of bacterial metabolic activity in monocultures as well as aquatic natural samples. Application of BONCAT to FCM has several advantages useful for future applications. Firstly, cell sorting of active cells, with the possibility of further analysis in environmental investigations is possible (Hatzenpichler et al., 2016; Couradeau et al., 2019). The active portion of a prokaryote community can be identified by 16S tagging (Hatzenpichler et al., 2016). A wide range of applications can be associated with cell sorting considering cells are not destroyed or damaged by the analysis. Couradeau et al. (2019)

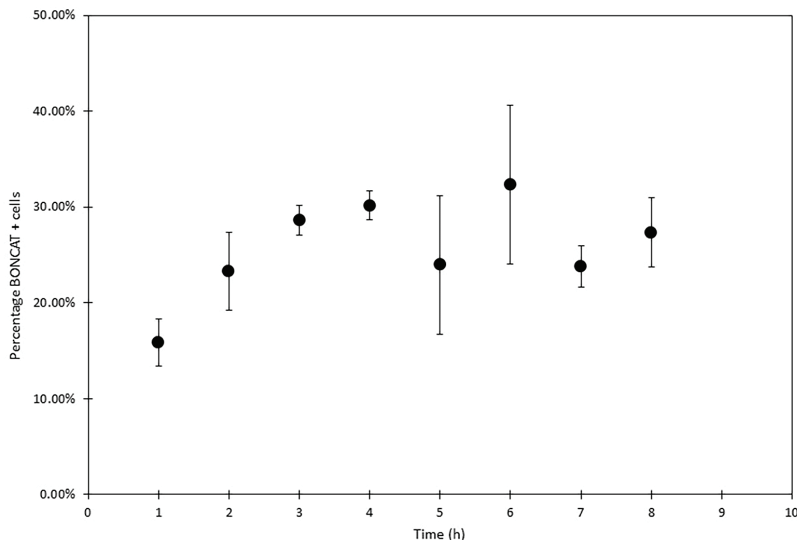

FIGURE 9 | Sea water microorganisms BONCAT analysis with FCM (SYBR Green and AF647 azide) over 8 h. Protein synthesis activity was monitored every hour (in triplicates biological samples and analyzed three times). Activity increased in the first three hours to reach a stable state.

compared BONCAT+ cells identified by 16S sequencing with bacterial isolates from the same environment. Pasulka et al. (2018) reported the possibility to observe virus production with BONCAT and microscopy analysis. Virus are produced from host cells proteins, and the incorporation of HPG thus enable the detection of newly produced viral particles. The use of SYBR Green as DNA stain is appropriate for virus analysis (Marie et al., 1999; Brussaard, 2004), and the adaptation of the protocol can be used to detect FCM BONCAT positive small particles that we interpret as viruses (**Figure 9**). Microbial growth to understand the impact of biotic and abiotic factors may also benefit of the BONCAT-FCM method (Sebastián et al., 2019). It is also possible to follow a bacterial community over time and identify principal actors and observe community changes in an environment associated with nutrients flux. If FCM instruments are available, onboard research vessels single cell activity can be monitored. Hatzenpichler defines BONCAT as part of the next-generation physiology approach where individual cells can be analyzed specifically (Hatzenpichler et al., 2020). The possibility to focus the research on active marine prokaryotes and not the whole community gives opportunity to improve or reinterpret microbial analysis.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

IAH, AL, OKH-E, and GB initiated and supervised the study. ML and GB performed the experimental part. ML wrote the manuscript, with significant inputs from GB, IAH, AL, and OKH-E. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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III

frontiers in Microbiology

ORIGINAL RESEARCH
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Flow Cytometric Analysis of Bacterial Protein Synthesis: Monitoring Vitality After Water Treatment

Mathilde Lindivat[1]*, *Gunnar Bratbak*[2], *Aud Larsen*[2,3], *Ole-Kristian Hess-Erga*[4] and *Ingunn Alne Hoell*[1]

[1] *Faculty of Engineering and Science, Institute of Safety, Chemistry and Biomedical Laboratory Sciences, Western Norway University of Applied Sciences, Haugesund, Norway,* [2] *Department of Biological Sciences, University of Bergen, Bergen, Norway,* [3] *NORCE Environment, NORCE Norwegian Research Center AS, Bergen, Norway,* [4] *Norwegian Institute for Water Research, Bergen, Norway*

Bacterial vitality after water disinfection treatment was investigated using bio-orthogonal non-canonical amino acid tagging (BONCAT) and flow cytometry (FCM). Protein synthesis activity and DNA integrity (BONCAT–SYBR Green) was monitored in *Escherichia coli* monocultures and in natural marine samples after UV irradiation (from 25 to 200 mJ/cm^2) and heat treatment (from 15 to 45 min at 55°C). UV irradiation of *E. coli* caused DNA degradation followed by the decrease in protein synthesis within a period of 24 h. Heat treatment affected both DNA integrity and protein synthesis immediately, with an increased effect over time. Results from the BONCAT method were compared with results from well-known methods such as plate counts (focusing on growth) and LIVE/DEAD^{TM} BacLight^{TM} (focusing on membrane permeability). The methods differed somewhat with respect to vitality levels detected in bacteria after the treatments, but the results were complementary and revealed that cells maintained metabolic activity and membrane integrity despite loss of cell division. Similarly, analysis of protein synthesis in marine bacteria with BONCAT displayed residual activity despite inability to grow or reproduce. Background controls (time zero blanks) prepared using different fixatives (formaldehyde, isopropanol, and acetic acid) and several different bacterial strains revealed that the BONCAT protocol still resulted in labeled, i.e., apparently active, cells. The reason for this is unclear and needs further investigation to be understood. Our results show that BONCAT and FCM can detect, enumerate, and differentiate bacterial cells after physical water treatments such as UV irradiation and heating. The method is reliable to enumerate and explore vitality of single cells, and a great advantage with BONCAT is that all proteins synthesized within cells are analyzed, compared to assays targeting specific elements such as enzyme activity.

Keywords: flow cytometry, BONCAT, bacteria, water analysis, vitality, UV irradiation, heat treatment

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***Correspondence:**
Mathilde Lindivat
Mathilde.lindivat@hvl.no

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INTRODUCTION

Determining bacterial physiological states is important for a wide range of applications. Hereafter, these physiological states are described as viable cells when bacteria have the capacity to reproduce (i.e., divide) and vital when they present metabolic activity and cell integrity but are unable to reproduce. Bacterial activity includes reactions, processes, and functions necessary for bacterial growth and development. Investigations into viability and metabolic activity of microorganisms

are necessary to assess their role in aquatic ecosystems (Czechowska et al., 2008). For industries that use bacteria in their production, bioprocess monitoring is important to control and maintain optimal conditions throughout the production (Rieseberg et al., 2001). In health research, the determination of effects of antimicrobial treatment is highly relevant to fight resistant bacterial pathogens (Léonard et al., 2016). Analysis of viable microbes in water is necessary to ensure safety and the absence of pathogens. Wastewater, drinking water, aquaculture, or ballast water onboard ships are all the examples where water treatment is required to prevent the spread of pathogens or non-indigenous species.

With such a broad spectrum of applications, a wide range of methods targeting different cellular processes has been developed during the last decades and applied to assess the physiological state and vitality of bacteria. Many of these are based on the use of fluorescent probes including the detection of DNA with permeable stains such as SYTO 9 and SYBR Green I; the control of membrane integrity with impermeable DNA stain propidium iodide (PI); analysis of membrane potential with the passage of charged molecules rhodamine 123 and DiOCx(x) derivate; efflux pump activity with the passive diffusion of small molecules such as ethidium bromide; and respiration activity and the electron chain transportation efficiency with cleavage of substrates INT [2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride] and CTC (5-cyano-2,3-ditolyl tetrazolium chloride) (Buysschaert et al., 2016). While developed for fluorescent microscopy, many of these methods have been adapted for flow cytometry (FCM) which often is considered both faster and cheaper (Hoefel et al., 2003). Interpreting staining data is, however, inherently difficult especially when methods are applied to analyze mixed and diverse natural communities (Shi et al., 2007; Hammes et al., 2011; Braissant et al., 2020).

Disinfection methods such as chlorination, ozonation, filtration, and UV irradiation, sometimes in combination, are used to inactivate bacteria (Pichel et al., 2019). To evaluate water disinfection and for compliance control, water microbial quality assessment is required to ensure that cells are removed, inactivated, or dead after treatment. Common methods include culturing and selective plating of pathogens (Allen et al., 2004), microscopy analysis (Dufour et al., 2003), detection of ATP (Lee et al., 2001), and a variety of PCR-based detection of specific pathogens (Ramírez-Castillo et al., 2015). FCM has also been shown to be an efficient instrument to analyze vitality of aquatic microbes (Hammes and Egli, 2010; Hoell et al., 2017; Safford and Bischel, 2019) and has been used for example to evaluate microbial counts in recirculating aquaculture farm (Rojas Tirado et al., 2018). In spite of this, FCM is rarely used as a standard method for water analysis (Safford and Bischel, 2019), perhaps due to the lack of multicolor-based method that can explore different cell parameters at the same time (Hammes and Egli, 2010).

Despite these aforementioned technological advances, the need remains for the development and improvement of methods to measure bacterial vitality and to evaluate water treatment strategies. Bio-orthogonal amino acid tagging (BONCAT) is promising in this respect and has been used for microbial analysis (Hatzenpichler and Orphan, 2015) and for routine analysis of natural marine microbial communities with FCM (Lindivat et al., 2020).

The principle is that alkyne- or azide-bearing amino acid analogs are incorporated during protein synthesis and subsequently labeled with a fluorescent dye by an azide–alkyne click chemistry reaction. BONCAT is efficient for determining bacterial vitality at the community level, but has, to our knowledge, never been used to assess the effect of physical or chemical disinfection procedures (such as UV, temperature, and H_2O_2) on bacterial activity. Since various treatments provoke changes in the bacterial metabolism to prevent death, determining whether BONCAT can be used as a reliable vitality indicator is necessary.

In this study, we assess the use of BONCAT in combination with FCM (BONCAT-FCM) as a method for evaluating different water disinfection treatments. The method was applied to monitor cell death and to determine vitality states (live, dead, damaged) in *Escherichia coli* monocultures and compared to plate counts and LIVE/DEAD^{TM} BacLight^{TM} staining. The vitality of bacteria from marine water samples after UV and heat treatments was also assessed with BONCAT-FCM.

MATERIALS AND METHODS

Bacterial Culture Maintenance and Environmental Samples

Monocultures of *E. coli* (ATCC 25922), *Aeromonas salmonicida* (ATCC 33658), *Listonella anguillarum* (ATCC 19264), *Yersinia ruckeri* (ATCC 29473), and *Bacillus cereus* (GMB 105.1, isolated from soil, Norway, provided by the University of Bergen, Department of Biosciences) were grown from frozen stocks on tryptic soy agar (BD Bioscience, United States) at 37, 20, 18, 26, and 30°C, respectively. For natural bacterial communities, surface seawater (<2 m) was collected with a sampling bottle in Haugesund harbor (Haugesund, Norway) and filtered with a 100-μm cell strainer filter (BD Bioscience, United States) to remove larger particles or organisms that could block the fluidic system of the flow cytometer.

Bacterial Disinfection: Experimental Setup

To evaluate the use of BONCAT for assessing the vitality state of bacteria in monocultures and natural aquatic samples, two common bacterial disinfection methods (UV irradiation and heat inactivation) were applied. The BONCAT results were compared with other vitality analysis methods [i.e., LIVE/DEAD^{TM} BacLight^{TM} (Invitrogen, Thermo Fisher Scientific, United States) and plate counts], and the experimental setup in **Figure 1** gives an overview of the different steps. Experiments were carried out three times with individual samples in triplicates.

A 10 ml of fresh liquid *E. coli* culture grown overnight (OD ∼1.0) was harvested by centrifugation at 16,000 × *g* (Multifuge 3SR+, Thermo Fisher Scientific, United States) for 5 min at room temperature (RT). The cells were washed 3 times with 1x PBS by

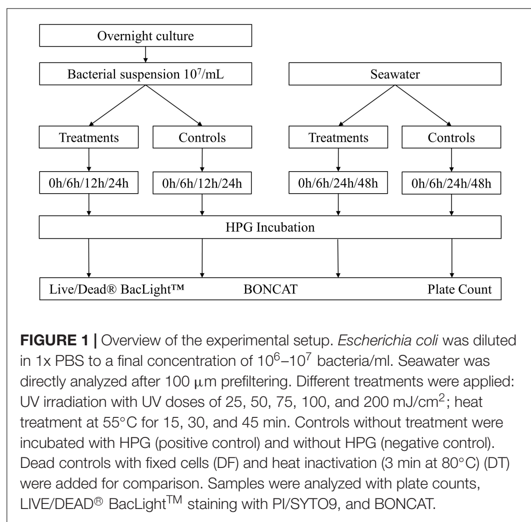

FIGURE 1 | Overview of the experimental setup. *Escherichia coli* was diluted in 1x PBS to a final concentration of 10^6–10^7 bacteria/ml. Seawater was directly analyzed after 100 μm prefiltering. Different treatments were applied: UV irradiation with UV doses of 25, 50, 75, 100, and 200 mJ/cm^2; heat treatment at 55°C for 15, 30, and 45 min. Controls without treatment were incubated with HPG (positive control) and without HPG (negative control). Dead controls with fixed cells (DF) and heat inactivation (3 min at 80°C) (DT) were added for comparison. Samples were analyzed with plate counts, LIVE/DEAD® BacLight^{TM} staining with PI/SYTO9, and BONCAT.

centrifugation and resuspension. The bacterial concentration was determined with SYBR Green staining and FCM (see below) and then diluted to a final concentration of 1×10^6–1×10^7 bacteria per ml in 45 ml of 1x PBS. Seawater samples were used directly without wash or dilution.

UV irradiation was performed with a collimated beam apparatus with two 150 W low pressure (LP) UV lamps (BestUV, Hazerswoude, Netherlands), according to recommendations by Bolton et al. (2015). Samples (45 ml) were irradiated in a sterile glass petri dish (diameter 6.7 cm, depth 1.28 cm) with magnetic stirring at 150 rpm (IKA Color Squid, Sigma Aldrich, United States). Calibration was performed with a radiometer (BestUV, Hazerswoude, Netherlands) at 254 nm. The distance between the UV lamp and the sample surface was 83.4 cm. Exposure times for the target fluences were calculated according to setup parameters (Bolton et al., 2015). Cells were exposed to UV irradiation for 2 min 20 s (25 mJ/cm^2), 4 min 40 s (50 mJ/cm^2), 7 min (75 mJ/cm^2), 9 min 20 s (100 mJ/cm^2), or 18 min 40 s (200 mJ/cm^2).

For heat treatments, samples were incubated at 55°C for 15, 30, or 45 min in a water bath (GD100, Grant instruments, United Kingdom), with regular manual mixing every 5 min. The PBS buffer used for bacterial dilutions was prewarmed to 55°C, to reach target temperature instantly.

Bacterial inactivation with various UV doses and heat inactivation of different lengths of time were compared to control samples; two untreated samples [with and without L-homopropargylglycine (HPG)], one dead control (dead formalin; DF) where the cells were killed with formaldehyde fixation (4% final concentration, 24 h before the experiment) and incubated with HPG, and another dead control (dead temperature; DT) where the cells were killed by heat treatment at 80°C for 3 min and incubated with HPG.

All samples were finally divided into four replicates of 10 ml, for later addition of HPG after 0, 6, 12, and 24 h of incubation for *E. coli,* and after 0, 6, 24, and 48 h of incubation for natural seawater samples. Incubation time was selected to observe direct and long-term effect of UV irradiation and heat on protein production and possible recovery effects. HPG 15 μM was added, followed by a 3 h of incubation at 37°C and 200 rpm (KS-10, Edmund Bühler, Germany) for *E. coli* and 6 h of incubation at 15°C and 200 rpm (KS-10, Edmund Bühler, Germany) for natural seawater, to allow incorporation of HPG during protein synthesis.

All samples were analyzed with BONCAT, LIVE/DEAD® BacLight^{TM} staining, and plate counts. The BONCAT samples (1 ml) were fixed with filtered formaldehyde (4% final concentration) and kept at 4°C until further analysis. The LIVE/DEAD samples were diluted and stained. Plate counts of *E. coli* and natural seawater bacteria were carried out by spreading 0.1 ml of diluted bacteria on tryptic soy agar (Difco, Becton Dickinson, United States) and 0.1 ml of natural seawater sample on marine agar (Difco, Becton Dickinson, United States), respectively. Plates were incubated for 48 h at 37°C for *E. coli* and 72 h at 15°C for natural samples.

Bacterial Vitality: Click Chemistry and LIVE/DEAD® BacLight™

Alexa Fluor 647 (AF647) in combination with SYBR Green DNA staining was used for all samples. Fixed samples (volume of ∼1.1 ml) were permeabilized using 1 ml 50% ethanol followed by 3 min of incubation at RT. Ethanol was removed after centrifugation 5 min at 16,000 × *g*. The subsequent incubation in 80 and 96% ethanol followed identical procedures. Samples were resuspended in 1 ml 1x PBS before the click reaction. First, a premix containing 5.75 μl of 20 mM $CuSO_4$ (Jena Bioscience, Germany), 11.5 μl of 50 mM Tris-[(1-hydroxypropyl-1*H*-1,2,3-triazol-4-yl) methyl] amine (THPTA) (Click Chemistry Tools, United States), and 0.3 μl of 10 mM AF647 dye (Click Chemistry Tools, United States) was incubated at RT in the dark for 3 min. Second, 57 μl of sodium ascorbate and 57 μl of aminoguanidine hydrochloride (Sigma Aldrich, United States) were added to each sample at a final concentration of 5 mM each. Finally, 17.5 μl of the premix was added to each sample. The samples were gently mixed by inverting the tubes before incubation at RT for 30 min in the dark. After incubation, samples were washed 3 times with 1x PBS buffer by centrifugation at 16,000 × *g*. Following the click reaction, samples were first diluted 10 times in a FCM tube with 1x PBS for *E. coli* or Tris-EDTA buffer (TE) for seawater, then double stained with 10 μl of 100X SYBR Green (final volume 1 ml) (Thermo Fisher scientific, United States), and incubated for 10 min in the dark prior to FCM analysis.

Bacterial vitality was also assessed with the LIVE/DEAD® BacLight^{TM} kit (Thermo Fisher Scientific, United States). The staining protocol followed the manufacturer recommendations. For all samples, 1.5 μl of PI (20 nM in DMSO) and 1.5 μl of SYTO9 (3.34 mM in DMSO) were added to samples diluted 10-fold (1 ml in 1x PBS) and incubated 15 min in the dark before FCM analysis. SYBR Green was used instead of SYTO9 for seawater samples.

Effect of Lethal Fixative Agents for Dead Control on BONCAT Signal

To evaluate the use of fixatives and possible unspecific background of BONCAT staining, we tested four different fixatives on five different bacteria and seawater. The samples analyzed were seawater samples and cell suspensions of *E. coli*, *A. salmonicida*, *L. anguillarum*, *Y. ruckeri,* and *B. cereus* in 1x PBS. Cell death was obtained by formaldehyde fixation overnight at 4°C (4% final), glutaraldehyde fixation overnight at 4°C (2.5% final), 70% isopropanol 30 min at RT, and 7% acetic acid 25 min at 30°C. A live positive control (with HPG) and a live negative control (without HPG) were included for comparison. Each sample was then incubated with 15 µM HPG for 3 h (6 h for seawater) and 200 rpm mixing (KS-10, Edmund Bühler, Germany) at each bacterium's respective growth temperature. Samples were subsequently fixed with formaldehyde (4% final) and stored at 4°C before continuing the click chemistry reaction.

Flow Cytometry

Initial bacterial cultures were counted by FCM after staining with SYBR Green I according to Marie and coworkers (Marie et al., 1999). In short, the cultures were diluted 1,000 times in 1x PBS (1 ml final volume), stained with 10 µl of 100X SYBR Green I (Thermo Fisher Scientific, United States), and incubated 10 min in the dark before FCM analysis. FCM analysis was carried out with an Attune NxT Acoustic Focusing Cytometer (Thermo Fisher scientific, United States) containing a violet laser 405 nm (50 mW), a blue laser 488 nm (50 mW), and a red laser 638 nm (100 mW). Instrument calibration was performed with Attune performance tracking beads (2.4 and 3.2 µm) (Thermo Fisher, United States). The following detectors were used for fluorescence detection: BL1 (530/30) for SYBR Green and SYTO9; BL3 (695/40) for PI; and RL1 (670/14) for AF647. Trigger was set at 2,000 on BL1 (SYBR Green and SYTO9). Compensation was performed for PI/SYTO9 according to the manufacturer's recommendations. Voltages were optimized for each detector. Between 500 and 10,000 cells were analyzed at a flow rate of 25 µl min^{-1}. LIVE/DEAD® BacLight^{TM} gating was performed as instructed by the manufacturer with live (intact membrane, SYTO9 fluorescence), dead (totally permeabilized membrane, PI fluorescence), and damaged (damaged membranes, SYTO9/PI fluorescence) cells. For BONCAT, cells were selected from SYBR Green or side scatter plots and back gated onto SYBR Green or AF647 plots (Lindivat et al., 2020). BONCAT-positive and BONCAT-negative gates were used to determine activity of marine microbes as described previously (Lindivat et al., 2020).

Statistical Analysis

Experiments were carried out in triplicates with individual triplicates for each sample. Paired *t*-test analyses were carried out to determine significant differences between controls and treated samples.

RESULTS

BONCAT Combined With DNA Staining as a Vitality Indicator

Results from the vitality assessment with BONCAT-FCM are shown in **Figures 2**, **3** and in **Supplementary Table 1** (replicate experiments gave similar results and are not presented).

Flow cytometry analysis was carried out on all treated samples (UV irradiation and heat treatment) and controls (with or without HPG). In addition to BONCAT, all samples were double-stained with SYBR Green. With this combination, the cells could be separated into quadrant gates (Q1–Q4) as described in **Table 1**.

The positive controls for *E. coli* showed a normal protein synthesis activity throughout the experiment, as demonstrated with 84–99% of active cell in Q2 (**Figure 2** and **Supplementary Table 1**). UV irradiation did not severely affect cells at 0 h as populations remained in Q2, similar to the positive control, but with a small decrease in SYBR Green intensity toward Q4 (**Figure 2** and **Supplementary Table 1**).

Effects of UV irradiation can be observed at 6 h with a decrease in fraction of live cells (Q2) and increase in damaged cells (Q4) (**Figure 2** and **Supplementary Table 1**). The fraction of inactive cells (Q1+Q3 = 0.9–2.7%, **Supplementary Table 1**) decreased, and we interpret this as a transient response to the transfer to PBS buffer and stable incubation conditions. Protein synthesis remained relatively high after 12–24 h with 26–60% live cells (Q2) and 34–60% of damaged cells (Q4). The percentage of inactive cells (Q1+Q3) varied from < 2% at 6 h to 14% at 24 h, demonstrating that cells maintain protein synthesis for a long period after UV irradiation at UV doses up to 100 mJ/cm^2. The exception to this is for UV dose of 200 mJ/cm^2 where 42 and 67% of the cells (Q1+Q3) had no protein synthesis after 6 and 24 h, respectively. UV-treated samples were significantly different from untreated controls ($p < 0.05$).

Heat treatment considerably affected cells immediately after treatment with 20–28% of damaged cell (Q4) (**Figure 3** and **Supplementary Table 1**). The proportion of inactive cells (Q1+Q3) increased from 2–3% at 6 h to 19–27% at 12 h. At 24 h, 59–68% of cells are severely damaged (Q4) and 24–33% are inactive with no BONCAT activity (Q3). Heat-treated samples were significantly different from untreated control samples ($p < 0.06$). In the heat-inactivated dead control (DT), 80–95% of the cells appeared as inactive (mainly in Q1). Low cell counts and the presence of cell debris causing high background noise in the FCM scatter plots precluded a more detailed analysis.

In general, cell concentration, as determined by FCM after staining with SYBR Green, remained around 1.5×10^6–4.5×10^6 cells/ml during the entire experiment for all untreated and UV-treated samples. However, cell concentration in heat-treated samples decreased compared to original concentration (7×10^5–9×10^5 cells/ml) most probably due to cell degradation.

Comparison of Vitality Methods: LIVE/DEAD BacLight and Plate Counts

Results from the LIVE/DEAD® BacLight^{TM} staining show live, damaged, and dead *E. coli* cells (**Figures 4A–C**) based on cell

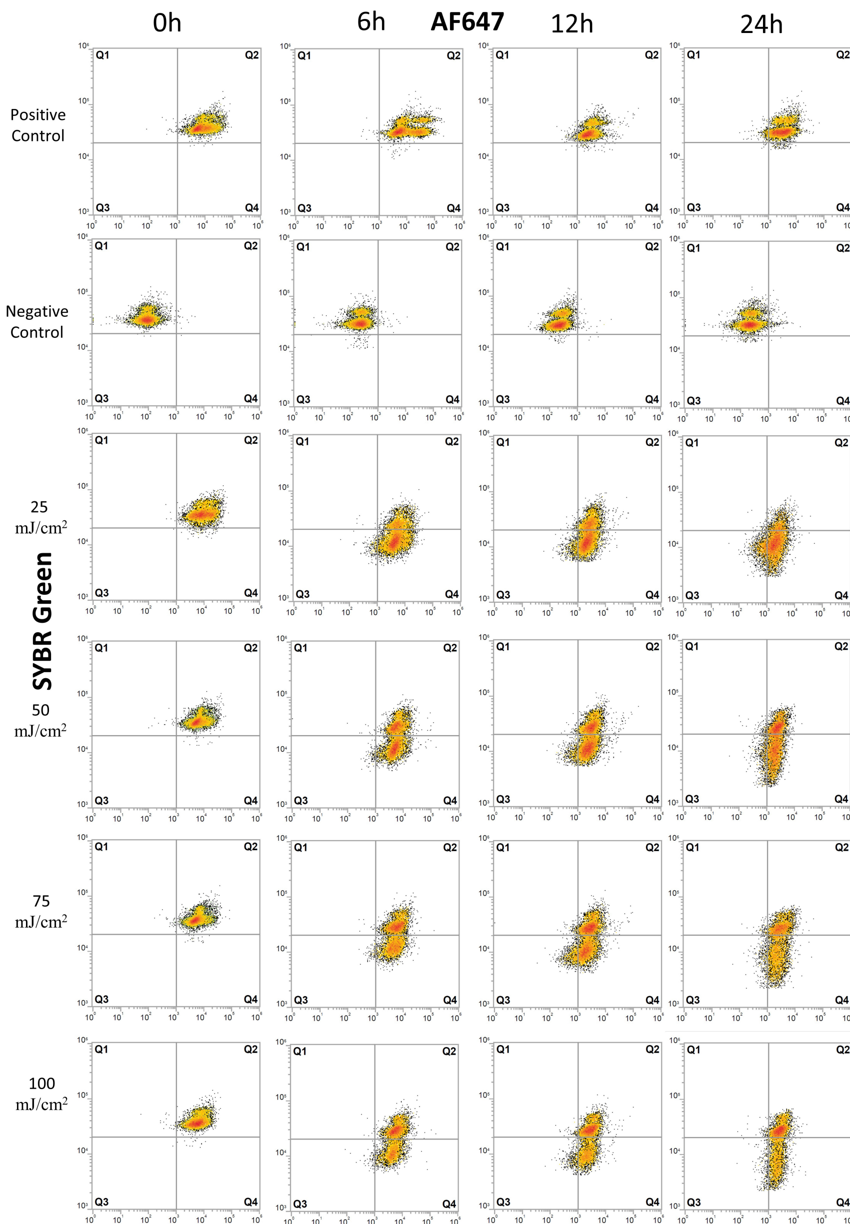

FIGURE 2 | Flow cytometry dot plots of *E. coli* cells treated with UV irradiation. Effects of UV doses of 25, 50, 75, and 100 mJ/m^2 on *E. coli* were monitored for degradation of DNA and for protein production at 0, 6, 12, and 24 h after treatment. Cells were stained with SYBR Green for DNA detection and AF647 for BONCAT. Between 5,000 and 10,000 cells were analyzed in each dot plot. Quadrant gates were designed from positive and negative HPG control with Q1: dead cells, the presence of intact DNA, negative BONCAT activity; Q2: live cells, the presence of intact DNA, positive BONCAT activity; Q3: dead cells, damaged DNA, negative BONCAT activity; and Q4: damaged cells with damaged DNA and positive BONCAT activity. The results of UV dose of 200 mJ/m^2 are not included as they present similar pattern as lower UV doses.

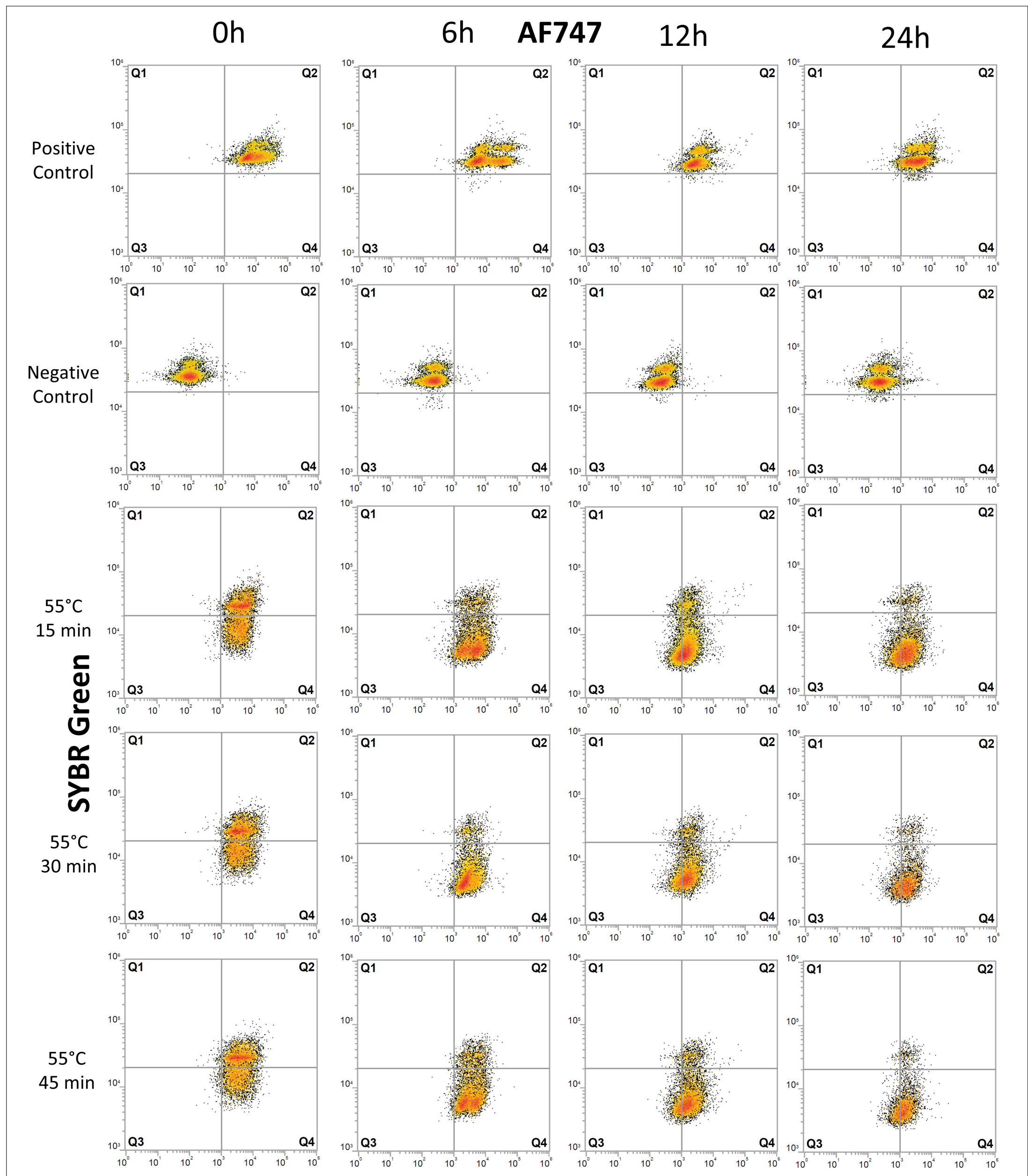

FIGURE 3 | Flow cytometry dot plots of *E. coli* cells treated with heat. The effects of exposure of *E. coli* cells to 55°C for 15, 30, and 45 min were monitored for degradation of DNA and for protein production at 0, 6, 12, and 24 h after treatment. The control samples are the same as those used in the UV experiment (**Figure 2**). Cells were stained with SYBR Green for DNA detection and AF647 for BONCAT. Between 5,000 and 10,000 cells were analyzed in each dot plot. Gates are identical to those used in **Figure 2**.

TABLE 1 | Overview of bacterial vitality states with BONCAT–SYBR Green.

Quadrant	SYBR Green	BONCAT	Physiological characteristics	Vitality
Q1	+	−	Intact DNA, no protein synthesis	Inactive
Q2	+	+	Intact DNA, protein synthesis	Live
Q3	−	−	Damaged DNA, no protein synthesis	Inactive
Q4	−	+	Damaged DNA, protein synthesis	Damaged

The different quadrants were obtained from FCM plots.
The presence of DNA was detected with SYBR Green and BONCAT with AF647.
Gates were determined from controls (positive and negative HPG).

membrane permeability. Loss of cell division based on plate counts is shown in **Figure 4D**. The controls showed that even without any treatment, the fraction of intact cells decreased and permeabilized cells increased, throughout the experiment (from 0 to 24 h) (**Figures 4A–C**). The several centrifugations to prepare the initial cell suspension can explain the gradual permeabilization over time as cell membrane can be damaged by collisions (Peterson et al., 2012). All UV doses caused permeabilization of cell membranes, with significant decrease in live cells after 6 h (**Figure 4A**). The percentage of damaged and dead cells increased accordingly (**Figures 4B,C**). The UV dose of 200 mJ/cm^2 permeabilized cells immediately (>90% damaged cells at 0 h) with the largest proportion of dead cells at 24 h (**Figures 4B,C**). Heat treatment significantly permeabilized cells membrane ($p < 0.004$) with only <4.5% of the cells remaining alive immediately after treatment (0 h) (**Figure 4A**). The percentage of live cells remained below 10% during the entire incubation period (**Figure 4A**). In the dead control sample (DT), the entire bacterial population was permeabilized and thus detected within the dead gate. Plate counts were compared with BONCAT and LIVE/DEAD® BacLight™ results for each sample (**Figure 4D**). Initial *E. coli* concentration was around 8×10^6–1×10^7 cells/mL for the controls (positive and negative). UV treatments and heat treatments significantly reduced cell concentration by 4–5 log and 5–6 log, respectively (**Figure 4D**). The UV dose of 25 mJ/cm^2 caused a decrease in *E. coli* CFU counts by four orders of magnitude (**Figure 4D**). Higher doses (50–100 mJ/cm^2) seem to inactivate all cells but the counts at 12 and 24 h suggest that some cells were not killed but able to revive and grow after the treatment. This regrowth can be explained by cell aggregation and shielding effects (Blatchley et al., 2001) in addition to release of nutrients by cell lysis at high UV doses (Villarino et al., 2003). No growth was observed at any timepoint when 200 mJ/cm^2 was applied suggesting that all cells were killed.

Assessment of Treatment for Marine Water Using BONCAT

Natural seawater samples were analyzed with FCM as described previously (Lindivat et al., 2020). For all natural seawater samples, including controls and UV treated samples, an increase in activity was observed after 6 h, which can be explained by the change

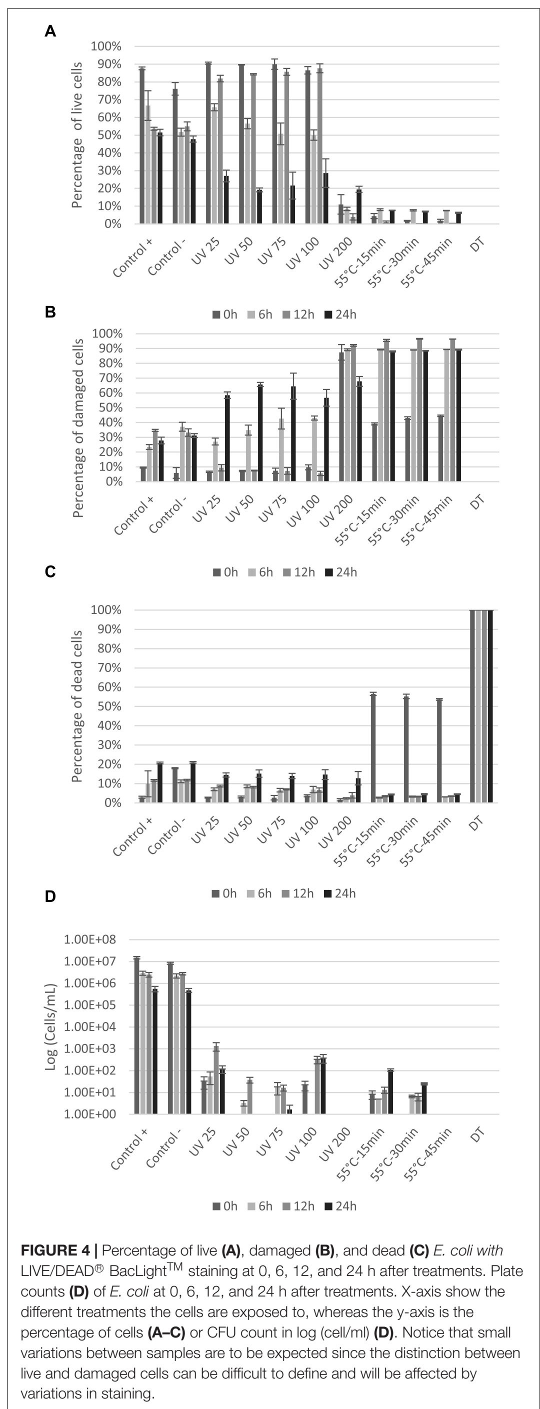

FIGURE 4 | Percentage of live **(A)**, damaged **(B)**, and dead **(C)** *E. coli with* LIVE/DEAD® BacLight™ staining at 0, 6, 12, and 24 h after treatments. Plate counts **(D)** of *E. coli* at 0, 6, 12, and 24 h after treatments. X-axis show the different treatments the cells are exposed to, whereas the y-axis is the percentage of cells **(A–C)** or CFU count in log (cell/ml) **(D)**. Notice that small variations between samples are to be expected since the distinction between live and damaged cells can be difficult to define and will be affected by variations in staining.

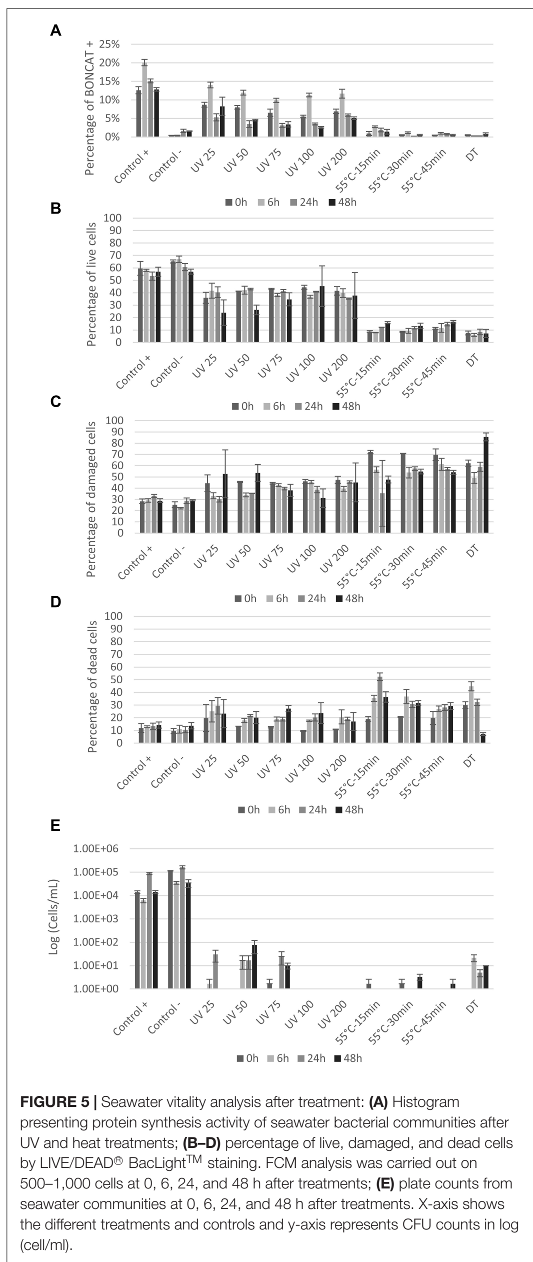

FIGURE 5 | Seawater vitality analysis after treatment: **(A)** Histogram presenting protein synthesis activity of seawater bacterial communities after UV and heat treatments; **(B–D)** percentage of live, damaged, and dead cells by LIVE/DEAD® BacLight^{TM} staining. FCM analysis was carried out on 500–1,000 cells at 0, 6, 24, and 48 h after treatments; **(E)** plate counts from seawater communities at 0, 6, 24, and 48 h after treatments. X-axis shows the different treatments and controls and y-axis represents CFU counts in log (cell/ml).

of environment from natural to laboratory-controlled conditions (Ferguson et al., 1984; Massana et al., 2001). The proportion of bacteria synthetizing proteins was close to 12 ± 2% at the beginning of the experiment (**Figure 5A**) and increased to 20% after 6 h to reach 12% at 48 h. UV treatments generally decreased this proportion below 5% after 24 h for UV doses of 50, 75, 100, and 200 mJ/cm^2 (**Figure 5A**). In general, the proportion of active bacteria decreased with time and increasing UV doses, except for dose of 200 mJ/cm^2. Heat treatment followed a similar trend with a decrease in the proportion of active cells over time after treatment and with prolonged treatment time. The fraction of active cells was significantly reduced below 2% at all timepoints (**Figure 5A**). Treated samples with UV and heat were significantly different from control samples ($p < 0.006$ and $p < 0.03$, respectively).

LIVE/DEAD® BacLight^{TM} kit gave somewhat different results compared to BONCAT (**Figures 5B–D**). For the controls, over 50% of the cells were detected as live, also over time after treatment. The percentage of damaged and dead cells was of 28–32% and 9–14%, respectively (**Figures 5C,D**). UV treatment caused a permeabilization of cells, but still between 24 and 45% of the cells remained live throughout the experiment (**Figure 5B**). Simultaneously, the number of damaged cells stayed stable (30–53%), but the proportion of dead cell increased from 9–19% at 0 h to 17–27% at 48 h (**Figures 5C,D**). The fact that all UV doses gave similar results indicates a similar cell response independently of the UV dose.

For heat treatments, cells were directly damaged (62–72%) with a slight increase in the proportion of dead cells after 6 h of incubation (27–36%) and a stabilization of the different populations at 24 and 48 h (**Figure 5D**). In comparison, plate counts showed limited growth after all treatments, but regrowth was observed for low UV doses (<75 mJ/cm^2) (**Figure 5E**). Some growth was observed in the dead temperature control (DT) meaning the treatment was not fully efficient for that experiment, and results from BONCAT and LIVE/DEAD® BacLight^{TM} kit conformed this observation (**Figure 5**).

The BONCAT laboratory protocol involves several centrifugation steps and is hence prone to cell loss (Lindivat et al., 2020). In BONCAT-FCM samples, the cell concentration was typically in the order of 10^4 cells/ml but as low as 10^3 cells/ml in heat-treated samples. In LIVE/DEAD® BacLight^{TM} samples, the concentration was 10^4–10^5 cells/ml. Comparison between treatments was nevertheless possible as we consider fractions and not absolute counts of cells.

Effect of Lethal Fixative Agents for Dead Control on BONCAT Signal

During the experiments with UV and temperature treatments, we applied a dead control (DF) consisting of cells fixed with formaldehyde. However, fixation of *E. coli* with formaldehyde surprisingly showed a BONCAT+ population comparable to a positive control. To investigate this phenomenon and test whether it is general or specifically related to *E. coli* and/or formaldehyde, we applied the BONCAT method to several different bacterial species and a natural marine bacterial community using different fixatives.

Fixation with glutaraldehyde gave autofluorescence in the red channel (overlapping with AF647 from the BONCAT staining protocol) and was therefore omitted from the results. Isopropanol

resulted in reduced protein synthesis in all samples, but the effect was variable and ranged from 17 to 90% residual activity (**Figures 6**, **7** and **Supplementary Figure 1**). Acetic acid and formaldehyde had little or no effect on protein synthesis in *E. coli, Y. ruckeri,* and *B. cereus* while for *A. salmonicida, L. anguillarum* and seawater samples, they resulted in substantial and complete inhibition respectively (**Figure 7**).

DISCUSSION

The combination of BONCAT and SYBR Green is a promising double staining technique for the assessment of water quality with respect to microbial activity. BONCAT has been used successfully to sort active vs. non-active bacteria in different environments and subsequently identify them (Hatzenpichler et al., 2016; Couradeau et al., 2019; Reichart et al., 2020). The method offers a new tool to evaluate vitality and activity of microorganisms (Emerson et al., 2017; Hatzenpichler et al., 2020). BONCAT was recently adapted to FCM for routine monitoring with the aim to enhance water microbial quality analysis (Lindivat et al., 2020). BONCAT follows protein synthesis activity *via* incorporation of the amino acid analog HPG. Previous studies of BONCAT showed similar incorporation results compared to radiolabelled [^{35}S]methionine and 3H-leucine, reinforcing the capacity of the method to follow protein synthesis in single bacterial cells (Samo et al., 2014; Leizeaga et al., 2017).

A wide range of vitality methods are available for FCM but so far, it is not possible to determine cell death directly (Davey and Guyot, 2020). BONCAT–SYBR Green can be used to distinguish live, damaged, and inactive bacterial cells based on their ability to synthetize proteins and DNA integrity (**Figures 2**, **3**), which are both essential to maintain metabolic activities, cell elongation, and division (Nebe-von-Caron et al., 2000).

The study of *E. coli* vitality with BONCAT after UV irradiation and heat treatment give information about single-cell metabolic activity over time. For the control samples, protein production stayed constant during the experiment. This is expected as stationary phase cells can maintain protein synthesis activity for over 60 h to extend cell longevity (Gefen et al., 2014; Jaishankar and Srivastava, 2017). The effect of UV irradiation was not immediately evident but significant after 6 h and there was apparently no dose-response. The heat treatment had in contrast an immediate impact with an increasing effect according to treatment length (**Figure 3**). This difference in cell response can be explained by the cellular targets of the treatments (e.g., DNA, proteins, and lipids) and treatment efficiency that will influence metabolic state and cell death (Davey and Guyot, 2020).

UV-C irradiations (254 nm) inhibit replication and transcription by damaging nucleic acids (DNA/RNA) and forming pyrimidine dimers and nucleic lesions (Oguma et al., 2002). UV irradiation as a water treatment technology has been extensively studied with different vitality methods (Hijnen et al., 2006; Safford and Bischel, 2019). UV-C irradiation between 4–500 mJ/cm^{-2} induces a reduction of ATP synthesis, esterase activity, membrane potential, and efflux activity (Villarino et al., 2000, 2003; Schenk et al., 2011). However, cell respiration is not affected by UV irradiation (Villarino et al., 2000; Blatchley et al., 2001; Guo et al., 2019). Villarino et al. (2000) even found residual esterase activity in *E. coli* for 48 h after UV treatment. We observed BONCAT activity in UV-irradiated samples even 24 h after treatment and despite major DNA damage (**Figure 2**). Villarino et al. analyzed protein synthesis after UV irradiation with the incorporation of [^{35}S]methionine and did not detect activity immediately after irradiation with UV dose of 10 and 80 mJ/cm^{-2} (Villarino et al., 2000, 2003). Our results from SYBR Green staining reveal DNA degradation as a main effect after UV irradiation, as shown in **Figure 2**. DNA degradation is then followed by a decreased but still present protein synthesis, which can be linked to metabolic activities and repair mechanisms for cell recovery. Two types of repair mechanisms are known, the photo repair and the dark repair (Goosen and Moolenaar, 2008). Since our samples were incubated in the dark, it is most likely the action of dark repair systems, such as excision base repair that removes damaged DNA segments (Jungfer et al., 2007).

In comparison, heat treatment had a greater impact on cell integrity and vitality. Heat treatment denatures proteins and liberate membrane components such as lipopolysaccharides but also intracellular enzymes (Tsuchido et al., 1985). We found an accelerated loss of protein synthesis and DNA content linked to treatment time. A similar inactivation has previously been observed for *E. coli* cells treated at 60°C for 30 min (Villarino et al., 2000) and at 85°C (Schenk et al., 2011) maintaining very little esterase activity and had compromised membranes.

Results from BONCAT were compared to well-known methods providing information about other cell functions than protein synthesis. LIVE/DEAD® BacLight™ stains cells with propidium iodide (PI) and SYTO9 to examine membrane permeability. PI enters dead or damaged cells and emits red fluorescence, whereas SYTO9 enters both live and dead cells, binds to DNA and RNA, and emits green fluorescence (Berney et al., 2007). BONCAT and LIVE/DEAD® BacLight™ thus target different cell functions and components and provide complementary information on cell physiology. The UV-treated cells lost their membrane integrity from 12 h, when protein synthesis was at its lowest (**Figures 2**, **4**). This is coherent with previous observations that loss of membrane integrity occurs in the late stage of cell death (Nebe-von-Caron et al., 2000; Berney et al., 2006; Hammes et al., 2011). On the other hand, heat treatment immediately impacted membrane permeability while ribosomes and DNA necessary for protein production were still functional (**Figures 3**, **4**).

BONCAT and LIVE/DEAD® BacLight™ vitality results did not correlate with the results from plate counts examining viability. Similar findings have been reported by others as cells still present activity without cell division (Blatchley et al., 2001; Kramer and Muranyi, 2014). Stressful conditions may induce a viable but non-culturable state (VBNC) that will allow cells to recover or persist despite not being able to divide (Colwell, 2000). VBNC cells maintain their integrity, a low metabolic

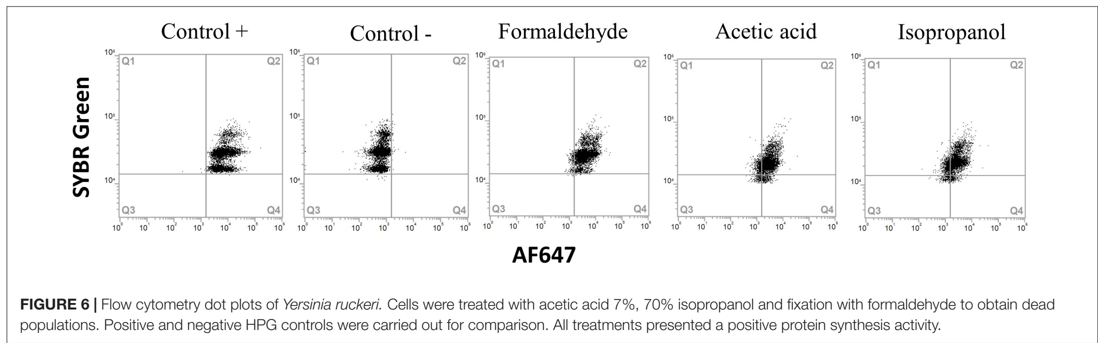

FIGURE 6 | Flow cytometry dot plots of *Yersinia ruckeri*. Cells were treated with acetic acid 7%, 70% isopropanol and fixation with formaldehyde to obtain dead populations. Positive and negative HPG controls were carried out for comparison. All treatments presented a positive protein synthesis activity.

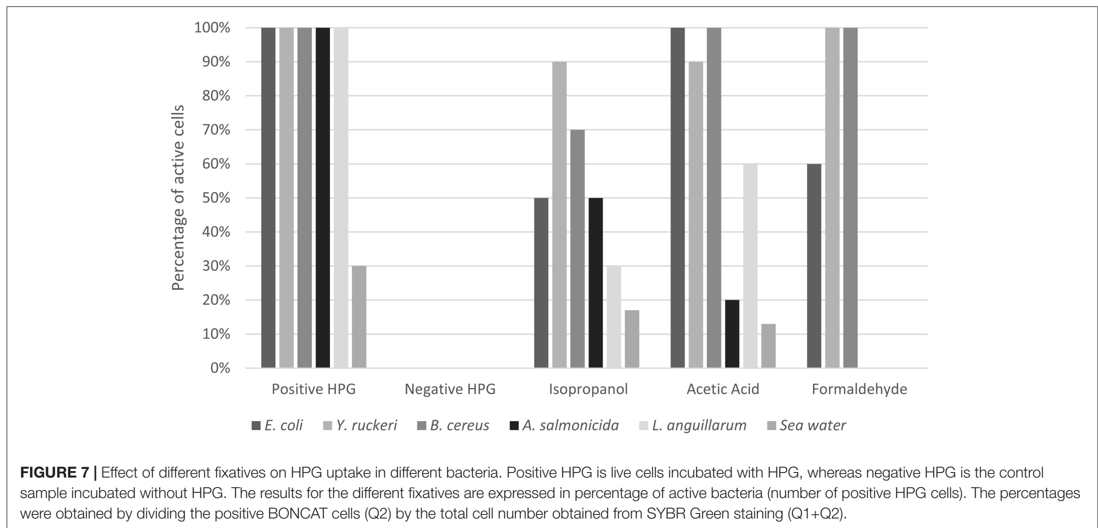

FIGURE 7 | Effect of different fixatives on HPG uptake in different bacteria. Positive HPG is live cells incubated with HPG, whereas negative HPG is the control sample incubated without HPG. The results for the different fixatives are expressed in percentage of active bacteria (number of positive HPG cells). The percentages were obtained by dividing the positive BONCAT cells (Q2) by the total cell number obtained from SYBR Green staining (Q1+Q2).

activity and gene expression (Ayrapetyan and Oliver, 2016). UV irradiation can induce this state, as previously shown for *E. coli* and *P. aeruginosa* (Zhang et al., 2015). Zhang showed that cells were able to resuscitate after low-dose UV irradiation (25 mJ/cm^2) (Zhang et al., 2015). Several studies have shown that VBNC cells that could not be detected with plate counts still exhibit physiological activity, demonstrated with enzyme activity with substrate CFDA (fluorescein diacetate derivate), membrane potential ($DIBAC_4$), and ATP assays (Berney et al., 2008; Kramer and Muranyi, 2014). We also observed similar results as cells were not able to divide on media but still maintained protein synthesis activity and also membrane integrity. Altogether, the three methods evaluate different aspects of cell physiology and provide information on vitality that are necessary to evaluate cell death. As cell death follows a pattern of disfunction ranging from loss of metabolic activity to membrane integrity (Davey, 2011), the three methods are complementary.

For environmental samples, the protein synthesis in general was low which is consistent with previous observation of marine samples analyzed with BONCAT (Lindivat et al., 2020). Marine bacteria display different levels of activity (e.g., inactive, slow growing, starving, dormant, active, and death), but inactivity does not necessarily mean dead (and similarly live does not always mean active) (Del Giorgio and Gasol, 2008). The reduction in protein synthesis related to increasing UV dose is consistent with results by Penru et al. (2013) that observed decreased cellular ATP concentration after irradiation.

For membrane permeability, we applied SYBR Green instead of SYTO9 as it is more used for environmental microorganisms (Marie et al., 1999; Berney et al., 2007) and distinction of live or dead cells was difficult to interpret as described previously (Shi et al., 2007). LIVE/DEAD® BacLight^{TM} staining with PI/SYBR Green showed a higher proportion (50–60%) of live cells than BONCAT (12–20%) for untreated samples. However, BONCAT and PI/SYBR Green showed a similar tendency for heat-treated cells where they lost metabolic activity and membrane activity immediately. Plate counts showed that both treatments efficiently reduced cell concentration. However, as cultivation

is not a reliable method to observe a total heterotrophic bacterial population, it was not possible to capture the whole microbial population compared to BONCAT and PI/SYBR Green (Allen et al., 2004).

The cells that were fixed with formaldehyde, even for more than 24 h, showed positive protein synthesis when incubated with HPG (**Figures 6**, **7**). One hypothesis to explain our results is that HPG, if not incorporated, somehow is trapped inside the cells during fixation and protein crosslinking, a process that take more than 24 h (Kiernan, 2000; Metz et al., 2004; Kamps et al., 2019). The observations that incubation with higher HPG concentration (25 vs. 5 μM tested with *E. coli* and *Y. ruckeri*) gave an increase in fluorescence and that adding methionine as a competitive amino acid to HPG when incubating *Y. ruckeri* and *A. salmonicida* did not eliminate the signal (data not shown) supports this hypothesis. There is to our knowledge no coherence (e.g., cell wall type, capsule formation) between the bacteria tested that may explain why they appear to behave differently. A more complete and specific study is necessary to verify this observation, and the possibility of other hypothesis should also be taken into account.

The observation that different fixatives had an inconsistent and incomplete effect on apparent protein synthesis in different bacteria concerns the validity of the BONCAT method (**Figure 7**). We have no reason to believe that inactive and truly dead cells retain or absorb HPG to yield false positives, and the effect of heat on *E. coli* shown in **Figure 3** supports this view. The significance of the phenomenon is, however, difficult to evaluate, for long incubation times and very active cells the relative amount of "false uptake" may be small, while for short incubations and less active cells, it may be important. Assuming that the false uptake comes to saturation after some time, it should be possible to correct for it by subtracting time zero blanks. For the results in this study, this means that the populations (i.e., dot plots) shown in **Figures 2**, **3** should have been further to the left but we would then have moved the quadrant gates accordingly and our interpretation and conclusions would not have been affected. Formaldehyde seems to be efficient for marine bacteria, and the seawater experiment shown in **Figure 6A** should hence be valid.

One advantage with BONCAT is that all proteins synthesized within cells are analyzed, compared to assays targeting specific elements such as enzyme activity with esterase substrates and respiration with CTC (Braissant et al., 2020). Combination of BONCAT with FCM reduced analysis time compared to microscopy (Lindivat et al., 2020). The method can detect, enumerate, and differentiate bacterial cells affected by UV and heat treatments. Compared to other vitality stains such as DNA stains or substrates that only requires incubation with the sample, BONCAT has many steps that are not compatible for routine analysis of microbial water quality. In addition, regulations for microbial water quality monitoring, e.g., for drinking water or ballast water, use culture-based techniques or PCR detection focusing on reproduction and detection of DNA instead of vitality (Figueras and Borrego, 2010; First and Drake, 2013). However, BONCAT has the capacity to determine microbial vitality for the development of water disinfection methods.

BONCAT is a promising method to determine vitality of bacterial cells after UV and heat treatment. BONCAT is complementary to other vitality staining methods such as LIVE/DEAD® BacLight™ and culturability. With proper calibration and incubation parameters, the method can be used to evaluate bacterial vitality in cultures and in natural samples.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

IAH, AL, OKH-E, and GB initiated and supervised the study. ML performed the experimental part. ML wrote the manuscript, with significant inputs from GB, IAH, AL, and OKH-E. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.772651/full#supplementary-material

Supplementary Figure 1 | Histograms of protein synthesis activity with BONCAT (AF647/RL1) for *Aeromonas salmonicida* (AS) and *Yersinia ruckeri* (YR). Cells were fixed with formaldehyde and incubated with or without 15 μM HPG for 3 h. FCM analysis was realized with SYBR Green/AF647 on 5,000–10,000 cells.

Supplementary Table 1 | Overview of the percentage of protein synthesis activity and intact DNA in *Escherichia coli* cells over time after treatment with UV irradiation and with heat. Calculations were carried out from BONCAT-FCM results. Quadrants were used as described in **Table 1**. FCM analysis was realized with SYBR Green/AF647 on 5,000–10,000 cells at 0, 6, 12, and 24 h after treatments.

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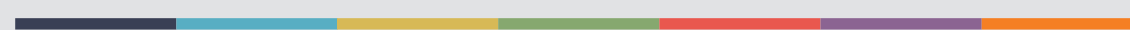

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