UNIVERSITY OF CALIFORNIA SAN DIEGO

Using BONCAT-FCM to Quantify Bacterial Production in Marine Observing Programs

A Thesis submitted in partial satisfaction of the requirements
for the degree Master of Science

in

Oceanography

by

Clay McClure

Committee in charge:

Professor Jeff Bowman, Chair
Professor Andrew Allen
Professor Lihini Aluwihare

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The Thesis of Clay McClure is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

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LIST OF ABBREVIATIONS

BONCAT	Bioorthogonal Noncanonical Amino-Acid Tagging
FCM	Flow Cytometry
AHA	Azidohomoalanine
NCAA	Noncanonical Amino Acid
BP	Bacterial Production
SPAAC	Strain Promoted Amino Acid Click Chemistry
SCCOOS	Southern California Coastal Ocean Observing System

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ABSTRACT OF THE THESIS

Using BONCAT-FCM to Quantify Bacterial Production in Marine Observing Programs

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Professor Jeff Bowman, Chair

Recent research has shown the potential of bioorthogonal noncanonical amino acid tagging (BONCAT) methods in studying microbial activity in marine environments in situ without the use of a radioisotope label. Click chemistry with fluorescent dyes in addition to BONCAT allows for the identification of the marine microbial community that actively synthesizes proteins. When used in this way, community fluorescence intensity relates directly to total protein synthesis and is thus a reasonable proxy for bacterial production. Here, we present a BONCAT protocol with flow cytometry (FCM) tested on the analysis of bacterial production across a broad

productivity gradient in the California Current ecosystem. Our method utilizes the non-canonical methionine substitute, L-azidohomoalanine (AHA) and strain-promoted click chemistry steps. The BONCAT-FCM method was first developed and tested on *Escherichia coli* cultures and coastal marine environmental samples. Samples processed using BONCAT-FCM were then directly compared to the ^{3}H-Leucine assay commonly used to estimate bacterial production on the California Current Ecosystem (CCE) P2107 process cruise. 210 samples were collected from varying depths over sampling cycles of three discrete water parcels. Similar bacterial activity trends were observed between the sampling cycles though the overall agreement between both protocols was weak. The results show the potential of BONCAT to act as a proxy for bacterial production in environments were radioisotopic labelling isn't feasible although more refinement of the BONCAT-FCM protocol is needed to reduce variability in measurements. Additionally, this method has greater potential for downstream analysis with fluorescence activated cell sorting (FACS) and subsequent genomic studies.

INTRODUCTION

Marine microbes are vital members of the oceanic food web. Decades of research has been dedicated to better describe the multi-faceted roles microbes occupy in the environment. One critical aspect of microbial ecosystem functions is in the balance between production and respiration in the global oceanic carbon chain. Investigating the activity of these marine bacterial populations has led to a deeper understanding of biogeochemical processes and carbon cycling in the ocean (Azam et al., 1994). Bacterial production (BP), the process by which heterotrophic marine bacteria assimilate fixed organic carbon from the environment for intracellular growth, emerged as a measurement for quantifying microbial activity (Azam et al., 1983). Historically, BP measurements of water column microbial communities have been collected using radioisotopic labeling with tritiated leucine or thymidine (Simon & Azam, 1992; Azam & Fuhrman, 1984). This method has proven effective in a wide range of environments and has remained a standard of measuring bacterial activity for decades. However, the radioactive nature of these techniques raises challenges. The use of radiotracers requires enhanced regulations and safety requirements that may restrict research in field studies (Cresswell et al., 2020). The advent of more advanced technology and chemistry has led to the development of novel methods for investigating marine microbial activity. Biorthogonal noncanonical amino acid tagging (BONCAT) has emerged as a particularly versatile and accessible tool for studying marine microbial activity.

BONCAT techniques have been developed to efficiently label newly translated proteins in bacterial populations without harming the cells (Dieterich et al., 2006). BONCAT methodology involves incubating target populations with synthetic amino acids *L*-homopropargylglycine (HPG) or *L*-azidohomoalanine (AHA) that exploit the promiscuous binding of methionyl-tRNA

synthetases to replace methionine in de novo protein synthesis (Dieterich et al., 2006) (Figure1.1). Newly synthesized proteins labeled with AHA or HPG can subsequently be visualized by conjugating fluorescent dyes to the synthetic amino acid using azide-alkyne click chemistry reactions (Best, 2009; MacGregor, 2014). Recent studies have proven the versatility of BONCAT methods to identify active portions of bacterial populations on a range of samples from clinical cystic fibrosis specimens to deep-sea marine sediments (Hatzenpichler et al., 2015; Krukenberg et al., 2021; Valentini et al., 2020). This work has established that BONCAT can be employed in significantly different environments and with taxonomically diverse communities of microorganisms. Thus, the potential of adapting BONCAT methods for use on water column microbes is feasible. The application of BONCAT to bacterial populations from marine water samples has already been successfully demonstrated in coastal surface waters and utilizing HPG and copper catalyzed click chemistry (CuAAC) (Lindivat et al., 2020; Samo et al., 2014). Additionally, some direct comparisons between BONCAT and ^{3}H-Leucine radioisotopic labelling have been performed in laboratory settings showing agreeability between the two methods (Leizeaga et al., 2017). However, a difficulty in utilizing BONCAT to measure BP similarly to radioisotopic labelling is how to quantify the BONCAT signal. Flow cytometry (FCM) analysis has been adapted for broad use in analyzing marine bacterial populations using fluorescent tagging (Monger & Landry, 1993; Petersen et al., 2012). The capability of high-throughput screening with FCM analysis allows for rapid enumeration of fluorescent signal and cell counts. Combining FCM methods to quantify BONCAT signals would lead to a numerical representation of bacterial activity from synthetic amino acid incorporation. After initial testing and method development, a BONCAT-FCM method was used on the California Current Ecosystem Long Term Ecological Research (CCE-LTER) P2107 Sampling cruise.

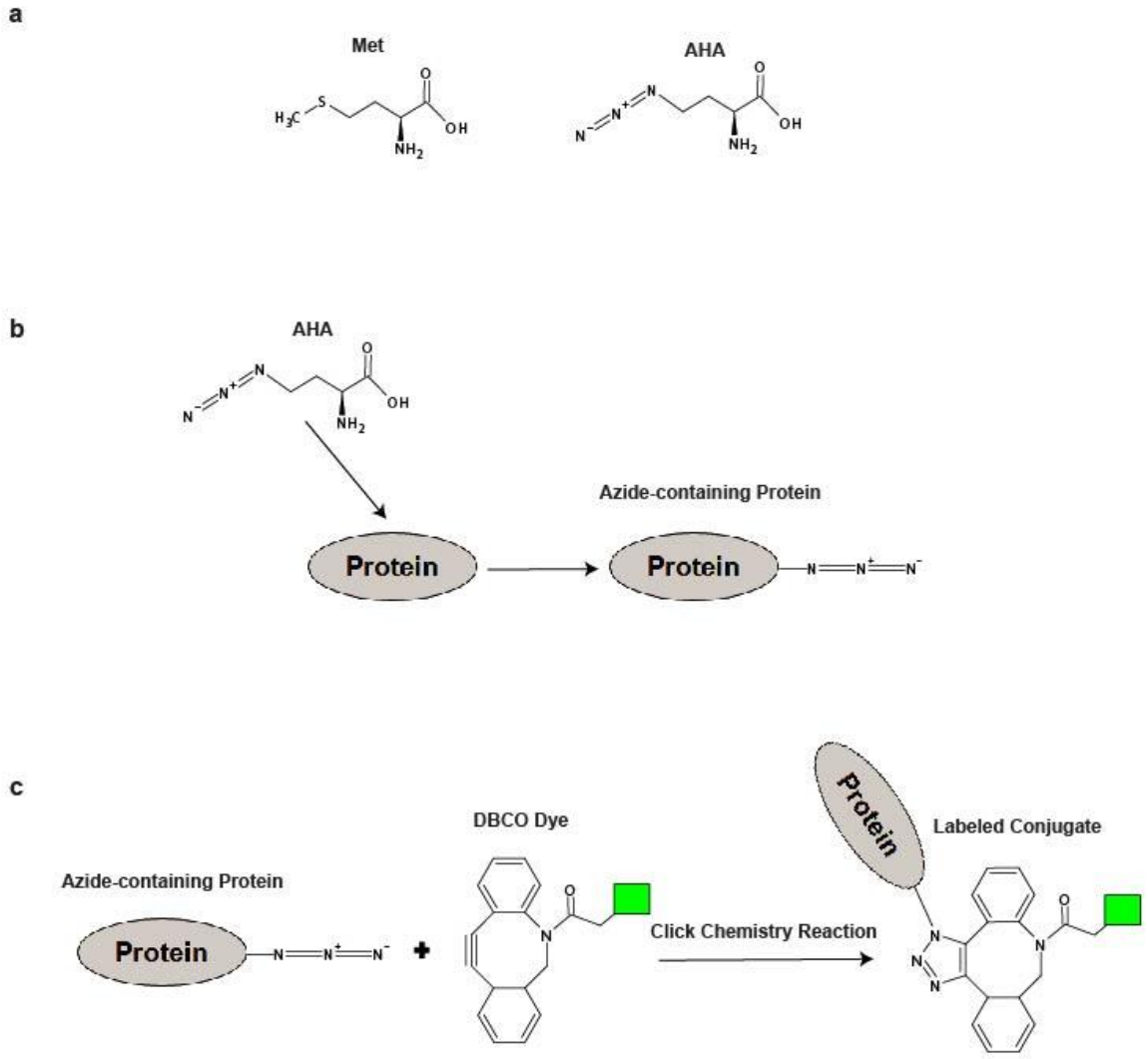


Figure 1.1: Overview of the BONCAT-FCM protocol with Strain Promoted Click Chemistry (SPAAC). Chemical structures of methionine (Met) and the noncanonical amino acid azidohomoalanine (AHA) are shown in (a). Visualization of AHA uptake and incorporation resulting in an azide-containing protein is shown in (b). Strain promoted click chemistry with the DBCO fluorescent dye resulting a labeled conjugate is shown in (c).

CHAPTER 1

1.1 BONCAT-FCM Testing with *Escherichia Coli*

The use of BONCAT with AHA combined with FCM analysis was originally tested on pure cultures of *Escherichia coli* to validate the method with bacterial populations. Much is known about the proteomics and activity of *E. coli* under controlled conditions, making *E. coli* a suitable candidate for BONCAT studies. Indeed, *E. coli* has been widely used as a model organism to test and develop BONCAT methodologies and has proven an effective system to study the effects of NCAA incubation and SPAAC fluorescent tagging (Hatzenpichler et al., 2014; Sherratt et al., 2017; Steward et al., 2020). The expanse of current knowledge on the proteome of *E. coli* under cultured and stressed conditions also limits the variability that experimental conditions could have on the results (Han & Lee, 2006; Schmidt et al., 2016; VanBogelen et al., 1997). Testing the method on a pure culture thus allowed for controlled experiments to explore the effectiveness and feasibility the BONCAT labelling method on bacterial cells.

The first testing of BONCAT-FCM methods on *E. coli* cultures resulted in low amounts (< 5%) of identified translationally active bacterial cells. This result was found to be caused by the original culturing methods of the *E. coli* monocultures using LB media. LB media is a nutritionally rich medium that includes methionine. In nutrient rich medias, cells preferentially uptake methionine over AHA resulting in much lower levels of AHA incorporation (Kramer et al., 2009). To enhance the uptake of AHA, cultures of K-12 *E. coli* were first grown on LB media to establish a healthy population then a sample was transferred to M9 minimal media. Transferring samples of the *E. coli* cultures to minimal media for a few hours before AHA labelling allowed for the depletion of intracellular methionine reserves to enhance the uptake of

AHA. Additionally, high amounts of background fluorescence in the fixed killed controls were observed in the first tests. These high levels of background fluorescence were most likely due to inefficient fixation of all the cultured bacterial cells. Increasing the time of paraformaldehyde fixation ensured that the killed controls were properly fixed to reduce the number of live cells diminishing the levels of background fluorescence.

Subsequent results from the testing of the BONCAT-FCM method with cultures of *E. coli* showed a shift in samples incubated with AHA compared to killed controls similar to findings reported from other studies (Hatzenpichler et al., 2014; Valentini et al., 2020). For the BONCAT-FCM testing with *E. coli* there was no DNA counterstain used so distinguishing the exact portion of the bacterial population that were identified by the BONCAT-FCM DBCO activity stain was difficult. It is also uncertain whether dead or lysed cells were present as false positives in the BONCAT samples. However, based on the event count of gated cells, the BONCAT treated samples had 7,561 identified active cells compared to the background level from the killed controls of 1,977 (Figure 1.2). Longer incubation times would result in a larger portion of identified active cells with a maximum of 100% of live cells but for the purposes of this validation testing, incubation times were kept short. Longer exposure (greater than 2 cell generations) to AHA increases the potential for the incorporated NCAA to cause detrimental effects to cellular function (Steward et al., 2020).

While testing the BONCAT-FCM method on pure cultures of bacteria is useful in investigating the general validity of BONCAT methods, environmental samples are much more diverse with more challenges in assessing the total community. The added variability of environmental conditions in marine samples can affect cellular activity significantly compared to pure cultures under controlled conditions. Furthermore, individual members in the marine

microbial community can have vastly different proteomes, meaning the degree of effectiveness of BONCAT at labelling active cells can vary among species, possibly excluding less-active species (Ngo et al., 2009; Tomanek, 2011). These factors highlight potential problems for BONCAT-FCM labelling of marine samples. A more in-depth testing of the BONCAT-FCM method was needed for environmental marine bacterial samples to better explore the validity of BONCAT-FCM under the variable conditions listed.

1.1.1 BONCAT-FCM Method for *ESCHERICHIA COLI*

Preliminary tests and validation of the BONCAT-FCM protocol were performed on *E. coli* cultures. Stock solutions of 100 mM *L*-azidohomoalanine (AHA) were prepared in pure DMSO, sterile filtered with 0.2 μm filters and stored at -20 ˚C. 10 mM working solutions were diluted using milliQ water and stored at -20 ˚C. Cultures of *E. coli* were grown in M9 minimal media at 37 ˚C overnight. Log growth phase of cultures was determined using a subsample and Synergy™ H1 microplate reader. 900 µL of *E. coli* culture in log growth phase was transferred to a 1 mL microcentrifuge tube. 100 µL of 10mM AHA was added to sample tubes. Samples were incubated at 37 ˚C for 2 hours. After incubation, samples were fixed with 4% paraformaldehyde in the dark at 4 ˚C for 24 hours. Killed controls were prepared by fixation first with 4% paraformaldehyde at 4 ˚C for 24 hours followed by 1mM AHA incubation at 37 ˚C for 2 hours. Samples were then either stored at -20 ˚C for future processing or processed by click chemistry for flow cytometry immediately following incubation and fixation.

(A) (B)

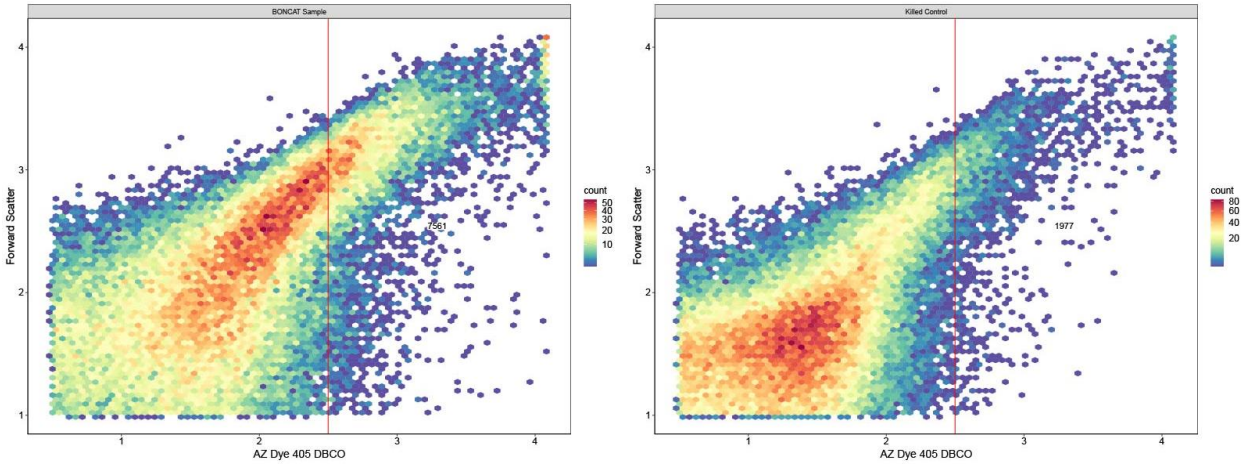


Figure 1.2: Density Scatter plots of BONCAT *Escherichia coli* samples (A) and killed control samples (B) obtained from flow cytometry. The red line represents the gate to distinguish cells identified as active from the BONCAT-FCM method.

1.2 BONCAT-FCM testing with Environmental Samples

Testing the BONCAT-FCM method on pure bacterial cultures of *E. coli* provided key insights in the validity of the technique in labeling bacterial populations. The results from pure culture testing provided enough framework to apply the BONCAT-FCM method to environmental marine samples. The coast of Southern California offers an ideal sampling area since many studies and time-series operate to understand the diverse microbial community in these productive waters. Diversity in environmental samples is one of many factors that can affect the labelling efficiency of the BONCAT-FCM method and raises the difficulty of accurately capturing an accurate representation of the active portion of the community (Samo et al., 2014). The reliable and consistent sampling from the Southern California Coastal Ocean Observing System (SCCOOS) has provided an immense amount of knowledge to better the understanding of the regional environment including the bacterial community (Terrill et al., 2006). Using the SCCOOS water sampling provided researchers with robust time series allowing for the description of seasonal patterns in the microbial community composition over time

(Mayali et al., 2010; Wilson et al., 2021). The robust work already completed using the SCCOOS sampling framework made it a useful choice for testing a BONCAT-FCM method on environmental samples.

Preliminary steps involved testing the BONCAT-FCM method developed for *E. coli* pure cultures directly on environmental samples. However, initial experiments showed that this method was not ideal for environmental samples. Since the optimal concentrations and incubation time for AHA supplementation differ among bacterial communities, a range of concentrations and incubation times was tested to find more optimal conditions for coastal environmental samples. Because the average growth rates and activity of marine bacteria sampled from the SCCOOS program will vary more than pure cultures of *E. coli*, the labelling percentage and efficiency was expected to be lower with environmental samples as reported in previous studies (Campbell et al., 2011; Marr, 1991).

A range of AHA concentrations (100 uM, 1M, and 6M) and incubation times (1 hr, 2hr, 3hr, 6hr, and 24hr) were tested based on previous studies to find BONCAT labeling conditions that suited the coastal marine environmental samples (Hatzenpichler et al., 2014; Leizeaga et al., 2017; Samo et al., 2014; Valentini et al., 2020). This optimization of AHA concentration and incubation time was carried out on environmental samples collected from the SCCOOS sampling program. Results from the active counts of bacteria determined by analysis of FCM data were compared using a linear mixed effects model. From the experiment, it was found that the incubation time had a significant effect on labelling efficiency with longer incubation times (≥ 3 hours) resulting in an increase in the identified active portion of bacterial communities in the water samples (Table 1.1, Figure 1.3).

Table 1.1: Statistical output of the linear mixed effect model used to analyze the results from the BONCAT-FCM AHA concentration and incubation time experiment for environmental water samples.

	Optimization of AHA Concentration and Incubation Time		
Predictors	*Estimates*	*CI*	*p*
Intercept	8.37	3.69 – 13.04	**0.001**
AHA Concentration (mM)	0.13	-0.23 – 0.48	0.481
Incubation Time (hr)	0.09	0.01 – 0.18	**0.025**
ICC	0.50		
$N_{Treatment}$	2		
Observations	72		
Marginal R^2 / Conditional R^2	0.039 / 0.518		

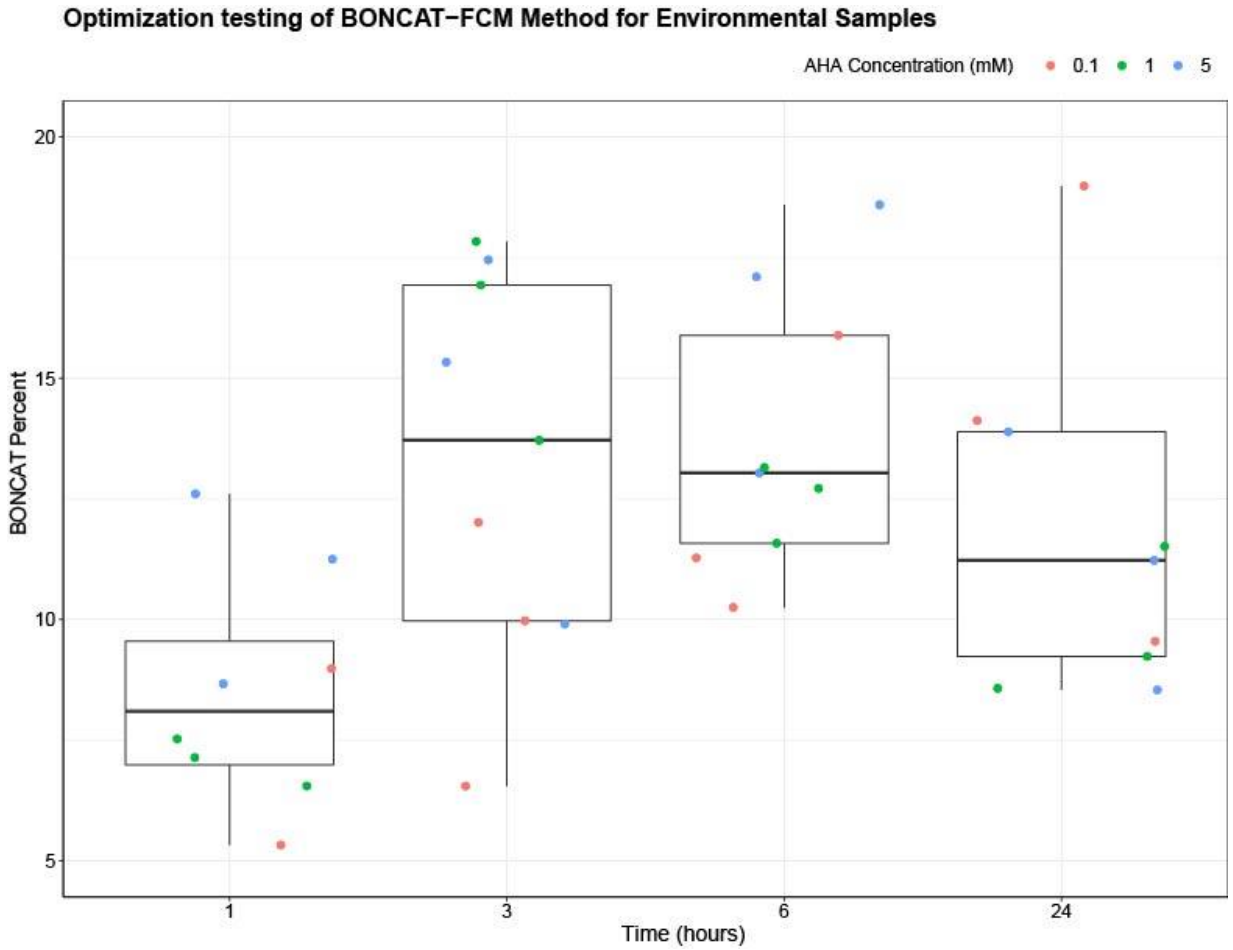


Figure 1.3: Boxplots of the percentage of active cells from the AHA concentration and incubation time optimization experiment on environmental water samples in triplicate. Percentage of active cells identified by FCM analysis. Colored scatter points correspond to the differing levels of AHA used for labelling across the four incubation time groups.

An incubation of three hours was chosen to examine further since limiting the amount of time bacterial communities are exposed to AHA will reduce the potential for negative effects caused by AHA incorporation (Steward et al., 2020). The concentration of AHA was found to not have a significant effect on BONCAT labelling efficiency. Upon further analysis, the higher concentrations of AHA (1mM and 5mM) had slightly lower labelling efficiencies than the lowest concentration of 0.1 mM (Figure 1.4). However, all samples exhibited better BONCAT labelling than the corresponding killed controls. Current hypotheses have asserted that the variability in BONCAT labelling due to species specific differences in activity among bacterial taxa could be reduced by using higher concentrations of NCAA (Samo et al., 2014). An AHA concentration of 1mM was thus chosen for the BONCAT-FCM method employed on the CCE P2107 sampling cruise. More experiments would have further helped in developing a BONCAT-FCM suited to environmental samples, however, issues with the SPAAC DBCO dye and time constraints during the testing window limited to amount of method development work possible.

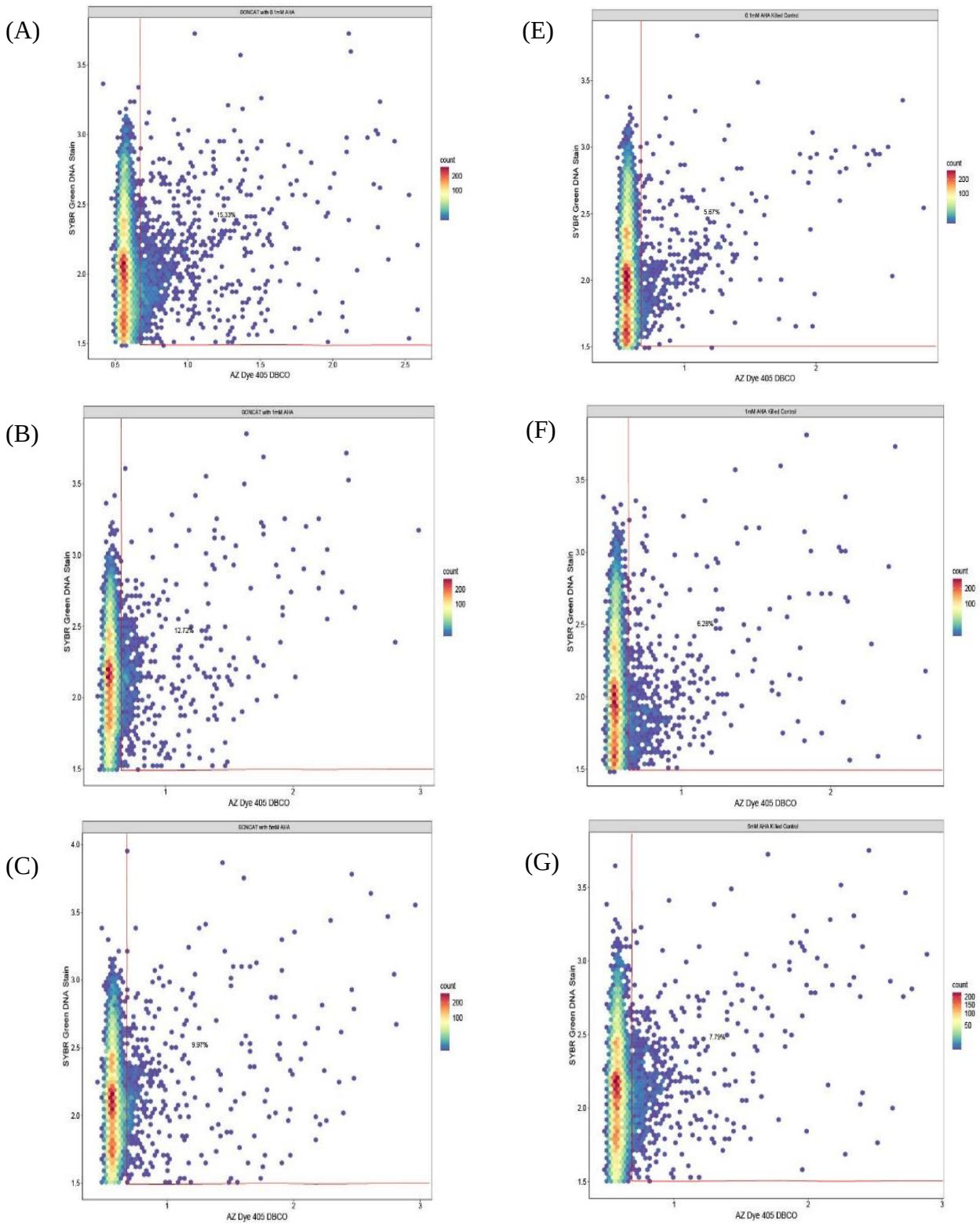


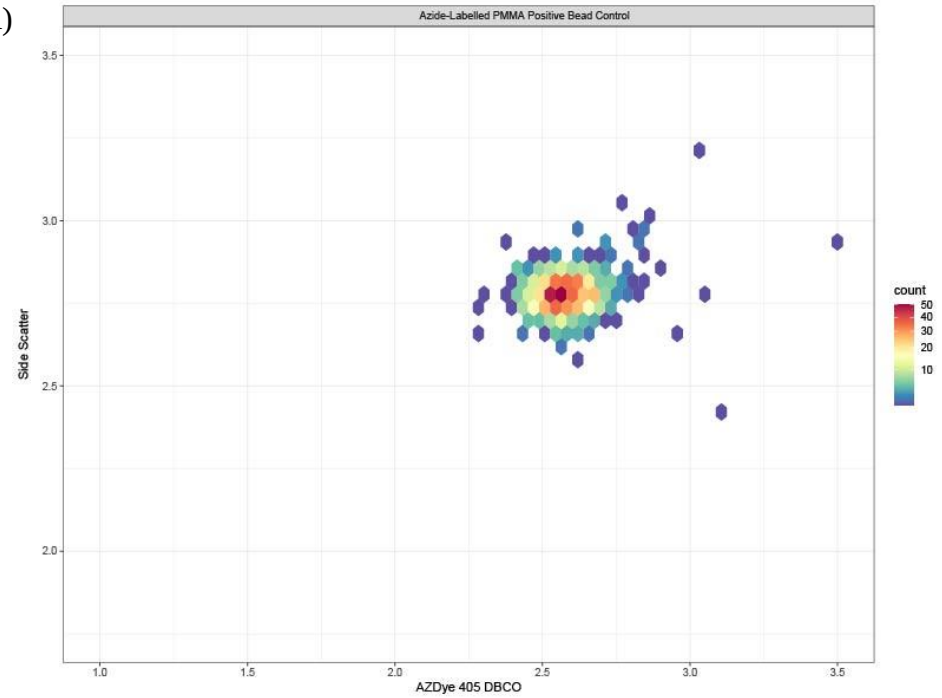
Figure 1.4: Density Scatter plots of BONCAT environmental samples (A,B,C) and killed control samples (E,F,G) from the 3-hour incubation experimental group with 0.1mM, 1mM, and 5mM AHA concentrations obtained from flow cytometry. The red line represents the gate to distinguish cells identified as active from the BONCAT-FCM method.

Along with testing different AHA concentrations and incubation times to optimize the BONCAT-FCM protocol, the use of positive control beads and centrifugal filter units allowed for further refinement of the protocol. The multiple centrifuging steps in the SPAAC protocol resulted in cell loss when using standard microcentrifuge tubes. Using centrifugal filter units resulted in much higher cell retention of the samples compared to regular microcentrifuge tubes (Table 1.2). Additionally, azide-labelled PPMA beads were used as positive controls to account for the variability between BONCAT samples using different aliquots of DBCO dyes. Positive bead controls also acted as a quality check to ensure the DBCO dye was binding adequately and emitting in the expected wavelength during FCM acquisition. These azide-labelled beads bind readily to the DBCO dye and produce consistent flow cytometry results (Figure 1.5). The average signal for all the positive bead controls was used as a correction factor for the CCE P2107 BONCAT samples fluorescent signal results. This correction factor was applied to all samples before the downstream analysis of the data following the sampling cruise.

Table 1.2: Recovered cell counts of BONCAT samples and killed controls after SPAAC washing steps using standard microcentrifuge tubes and centrifugal filter units (centricons).

Treatment	Tube Type	Amount of Stock Culture	Average Cell Count (FCM count)
BONCAT	1.5 mL Microcentrifuge Tube	1 mL of Sample	40,000-50,000 cells
BONCAT	Centricons	500 uL of Sample	40,000-50,000 cells
Killed Control	1.5 mL Microcentrifuge Tube	1 mL of Sample	30,000-40,000 cells
Killed Control	Centricons	500 uL of Sample	20,000-30,000 cells

(A)



(B)

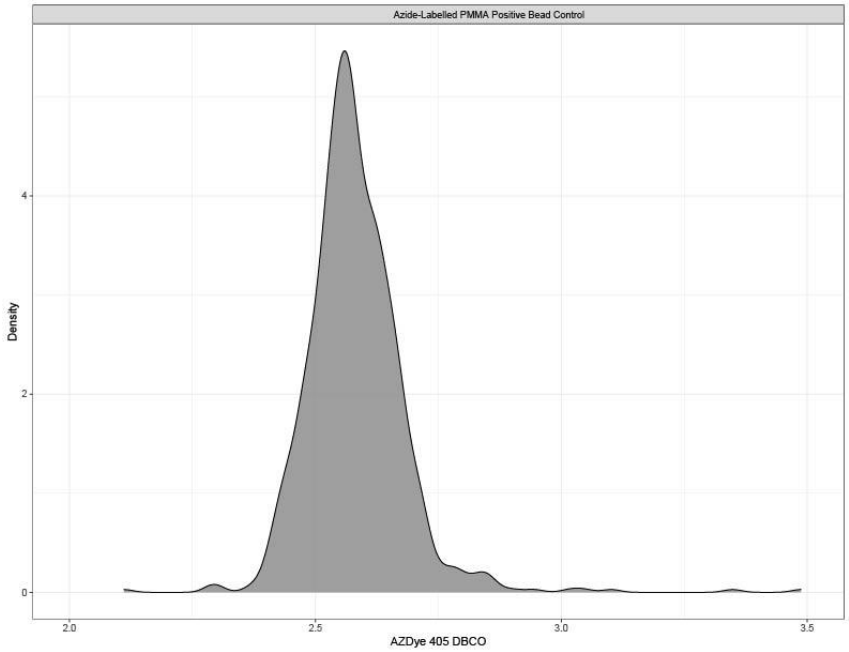


Figure 1.5: Density Scatter plots from FCM of azide-labelled PMMA positive bead controls (A) and corresponding density histograms of fluorescence (B).

1.2.1 BONCAT FCM Method for Environmental Samples

Marine water samples for testing were collected by the Southern California Coastal Ocean Observing System (SCCOOS) program from the Scripps Ellen Browning Scripps Memorial Pier. Marine water samples were filtered through 60 µM filters to prevent instrument clogging. 450 µL of pier water was added to 500 µL centricon sample tubes. Amicon Ultra Centrifugal Filter Unit 100 KDa sample tubes were used to minimize cell loss throughout the protocol. Samples were incubated in triplicate with final AHA concentrations of 6mM, 1mM, and .1mM. Triplicate samples at each concentration were then incubated for 1, 3, 6, or 24 hours at 18 ˚C. After AHA incubation, samples were fixed with 4% PFA for 2 hours in the dark at 4 ˚C. Negative killed controls in triplicate were fixed with 4% PFA first and then incubated with AHA.

CHAPTER 2

2.1 BONCAT-FCM and ^{3}H-Leucine Radioisotopic Method Comparison on Marine Samples

The initial testing of the BONCAT-FCM method with AHA and SPAAC on pure cultures of *E. coli* and environmental samples was necessary to develop a method more suited to the variable environment of the Pacific Ocean near the Southern Californian coastline. All environmental samples for the BONCAT-FCM method development were collected from coastal surface waters. However, bacterial production varies significantly throughout the water column and under different nutrient conditions (Chin-Leo & Kirchman, 1988; Ducklow & Carlson, 1992). For a true comparison of BONCAT-FCM to current BP measurements using ^{3}H-Leucine radioisotopic labelling, environmental samples across a nutrient gradient and depth profile were needed to accurately test the validity of the BONCAT-FCM method. The CCE-LTER P2107 process cruise provided a key opportunity to test both methods on a diverse set of environmental samples collected from Pacific Ocean waters near the southern Californian coast.

In this study, we explore the potential of BONCAT with FCM analysis to obtain a measurement of BP in coastal California waters. The BONCAT method was adapted to use AHA and copper-free click chemistry (SPAAC). This BONCAT-FCM method is employed on 210 environmental samples collected as part of the California Current Ecosystem Long-Term Ecological Research project (CCE-LTER). ^{3}H-Leucine radioisotopic labeling was also performed on a matched set of 210 environmental samples for direct comparison between the two methods. The sample collection represents a diverse range of nutrient and primary production gradients across the three sampling cycles of the CCE-LTER P2107 process cruise.

2.2 Methods

2.2.1 Cruise Sample BONCAT Protocol

A modified version of the BONCAT protocol from (Hatzenpichler & Orphan, 2016) was used on the P2107 process cruise for the California Current Ecosystem Long-Term Ecological Research program (CCE-LTER) from July to August of 2021 on the R/V Roger Revelle. Marine water samples for BONCAT analysis were taken from the midday CTD cast during the three cruise sampling cycles. Samples were taken at multiple different depths throughout the water column on each cast. In the ship-board laboratory, 5 mL of water was filtered through a 60 µm Nylon net filters (NY6002500 Millipore). 450 µL of filtered water was added to 1.5 mL centrifuge tubes in triplicate with one killed control.

L-azidohomoalanine (AHA) (Click Chemistry Tools, USA) 100mM stock solution was made by dissolving AHA in DMSO and filter sterilized through 0.2 µm filters and stored at -20 ˚C. Working stock solutions of AHA were prepared by diluting with MilliQ water to 10mM. Samples were incubated with a final AHA concentration of 1mM. All samples were incubated with AHA for 3 hours in the dark at room temperature (~21 ˚C). After AHA incubation, samples were fixed with 0.2 µm-filtered formaldehyde (Sigma Aldrich, USA) to a final concentration of 4% for 2 hours at 4 ˚C. After fixation, BONCAT samples were frozen and stored at -80 ˚C for the remainder of the cruise. For killed control samples, the fixation incubation was preformed first followed by the AHA incubation.

2.2.2 ^{3}H-Leucine Protocol

Samples for comparison were collected on the P2107 research cruise using the ^{3}H-leucine incubation method for measuring bacterial production from (FarooqAZAM, n.d., p. 1). Marine

water samples were collected from the same midday cast as the BONCAT samples. In the ship-board radiation laboratory, 1.7 mL of water samples was transferred to screw cap tubes in triplicate. L-[4,5-^{3}H] leucine (Moravek Inc. MT-672, Molar Activity = 29.8 Ci/mmol) was added to the samples to a final concentration of 20 nM. Samples were incubated with ^{3}H-leucine for 1 hour at room temperature (~21 ˚C). After incubation, samples were fixed with trichloroacetic acid (TCA) (5% v/v final concentration). Killed controls were fixed with TCA first and subsequently incubated with ^{3}H-leucine.

After incubations, all samples were pelleted using centrifugation (16,000 g, 10 min, 4 ˚C) and washed with 5% TCA followed by a wash with 80% ethanol. After wash steps, supernatant was removed from the pelleted samples to leave as little liquid in the vials as feasible. Sample pellets were frozen and stored at -20 ˚C to await further processing. When back the land-based laboratory, sample pellets were thawed overnight. Scintillation cocktail was added to each sample tubes and samples were incubated in the dark at 4 ˚C. After at least 24 hours of incubation, sample activity was collected using a scintillation counter. Estimates of carbon production were calculated using the 3.1 kgC/mol conversion factor found in Simon & Azam (1989).

2.2.3 Strain Promoted Click Chemistry

An optimized protocol for strain promoted click chemistry (SPAAC) was developed for the BONCAT samples collected on the P2107 process cruise. Collected samples were stored at -80 ˚C until ready for processing. Prior to SPAAC, samples were thawed overnight at 4 ˚C in the dark. Once thawed, samples were transferred to a 500 µL centricon tube (Amicon Ultra Centrifugal Filter Unit 100 KDa, UFC510024 Millipore) to prevent cell loss. Samples were

pelleted via centrifugation (5,000 g for 10 minutes) and permeabilized with 0.05% Tween-20 in 1X PBS for 3 minutes.

After permeabilization, samples were pelleted via centrifugation and washed with 1X PBS. Samples were then resuspended in freshly made 2-chloroacetamide (100 mM) and incubated for 1 h at 46 ˚C in the dark to block free thiols. After 1 h, AZDye ™ 405 DBCO (Click Chemistry Tools, 1 mM stock stored at -20 ˚C) was added to samples at a final concentration of 1 µM. Samples were incubated for 30 minutes at 46 ℃ in the dark. After click chemistry incubations, samples were washed to remove excess dye. All samples were pelleted via centrifugation and washed once with 50% EtOH followed by three more washes with 1X PBS, centrifuging samples between each wash. Samples were then resuspended in 1X PBS and 200 µL of sample was plated in a 96-well plate. Samples where then counterstained with SYBR™ Green I Nucleic Acid stain (ThermoFisher Scientific) prior to flow cytometry analysis.

Positive controls were made using DiaPoly Azide Poly (Methyl Methacrylate) PPMA beads (DNP-PA088, Creative Diagnostics) diluted 1:100 in 1X PBS. Positive controls were incubated with AZDye ™ 405 DBCO for 30 minutes at 46 ℃ in the dark with the other samples. The positive bead controls then underwent the same wash steps as the samples.

2.2.4 Flow Cytometry Analysis

BONCAT labelled samples and controls were analyzed on a Guava Easycyte Desktop Flow Cytometer (Luminex) equipped with a 405 nm Violet laser and a 488 nm Blue laser. The GRN-B (525/30) detection channel was used to detect SYBR Green stained events, and the BLU-V (448/50) channel was used to detect AZDye 405 DBCO. The InCyte™ software was used to collect the event data for the samples. A cutoff of 20 was used on the GRN-B-HLog channel to

remove excess noise seen in preliminary analysis. Plated samples were analyzed on the flow cytometry using a flow rate of 0.12 $\mu L \cdot s^{-1}$ and a collection time of 120 seconds. About 14.10 µL of each sample was analyzed.

2.2 Results

2.3.1 BONCAT-FCM identification of active Marine Bacteria

The modified BONCAT-FCM method described here was applied to marine environmental water samples from a range of depths and locations along the CCE-LTER study area. Samples were incubated with AHA and fluorescently labelled by strain-promoted click chemistry with AZDye 405 DBCO activity stain. To distinguish bacterial populations in the FCM analysis, samples were counterstained with SYBR Green I DNA stain. Results were corrected using the positive bead controls to reduce the inter-variability between samples prior to gating and analysis. The results from flow cytometry analysis show a clear difference between BONCAT samples and killed control samples (Figure 2.1). The active portion of the marine bacterial samples was found to be up to 27% of identified cells (Figure 2.1a). Activity gates were applied manually to preserve the most labelled events while reducing the amount of background noise captured.

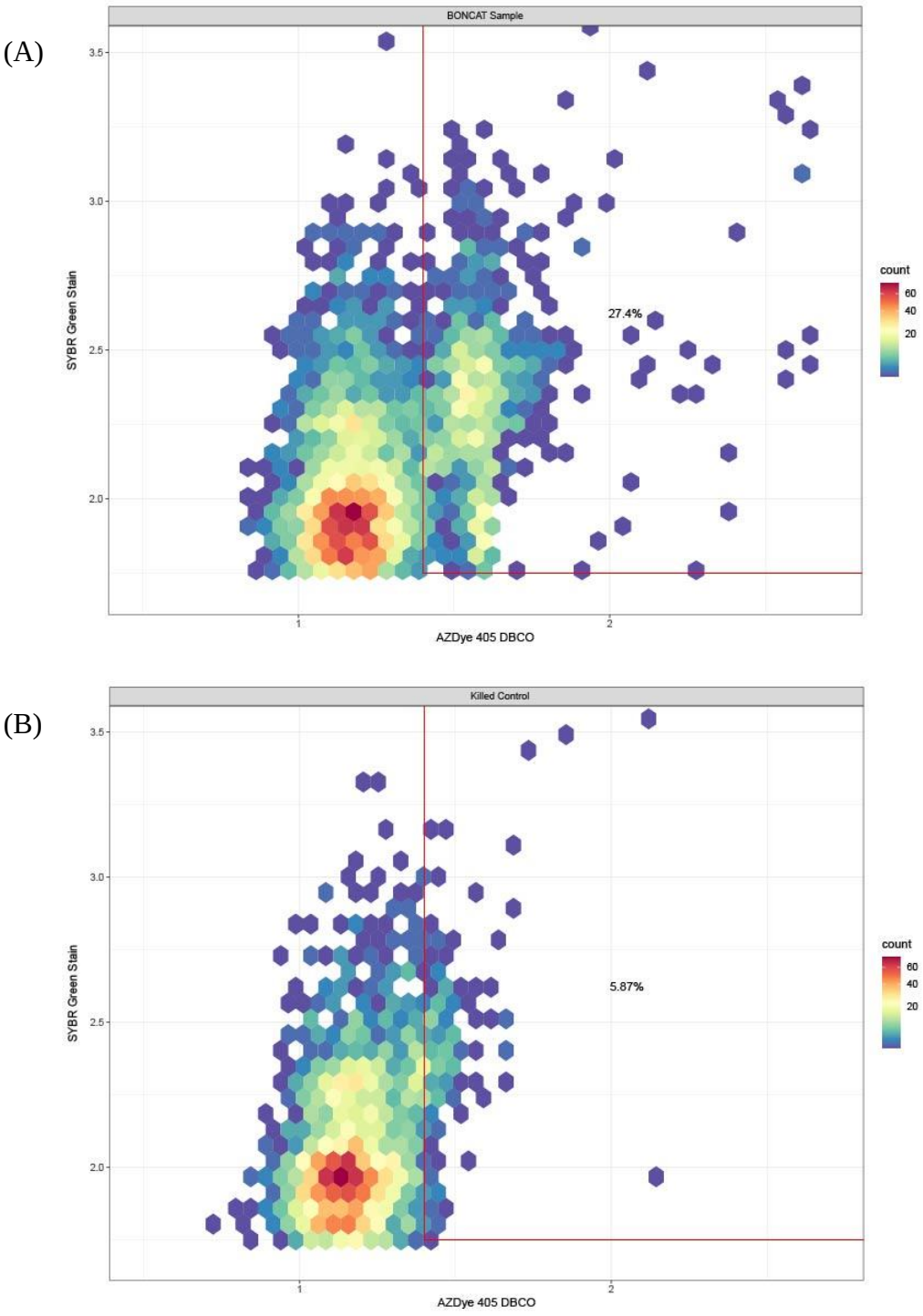


Figure 2.1: Density Scatter plots of BONCAT samples from the CCE P2107 Process cruise (A) and killed control samples (B) obtained from flow cytometry. The red line represents the gate to distinguish cells identified as active from the BONCAT-FCM method. The identified percentage of events in the gated region is displayed.

A quantification of bulk fluorescence was obtained by summing together all the BONCAT signal fluorescence events from the BLU-V (448/50) channel corresponding to the AZDye 405 DBCO activity stain. Signals for the BONCAT samples exhibited a second peak separate from the main population indicating the active portion of the sample (Figure 2.2). This peak was mostly absent from the killed control samples. The bulk fluorescence signal calculated for the killed controls was subtracted from the BONCAT samples to get a numerical estimate of bacterial activity captured with the BONCAT-FCM protocol.

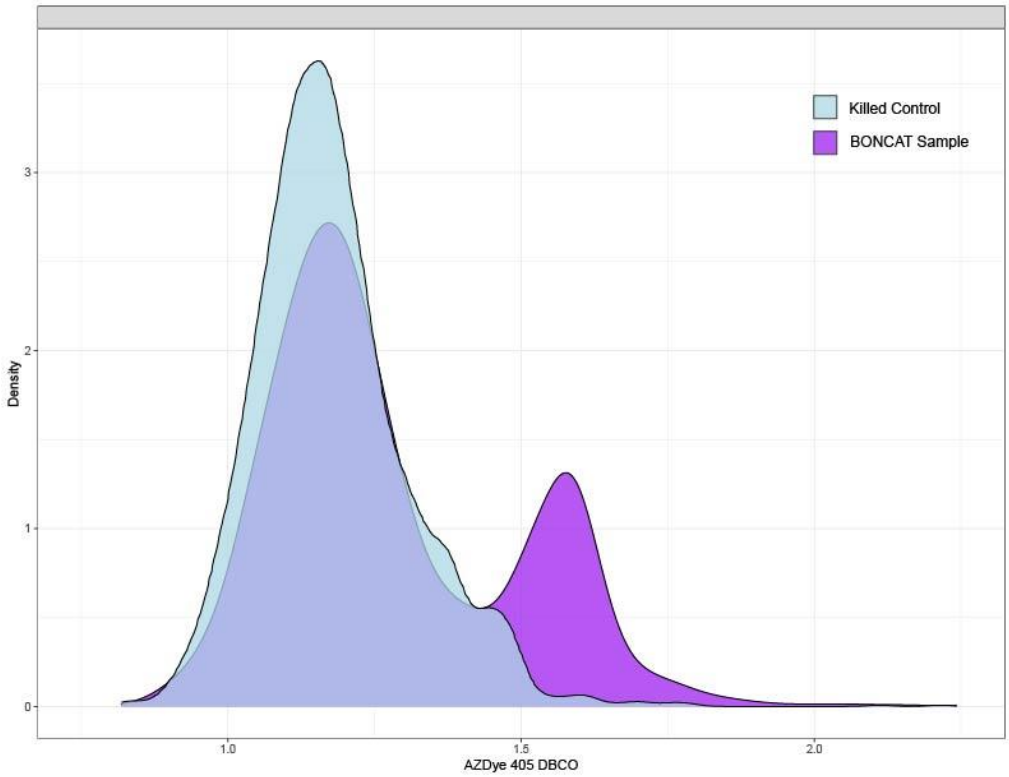


Figure 2.2: Stacked density histograms of CCE P2107 BONCAT sample fluorescence (purple) from FCM analysis compared to killed control samples (light blue).

2.3.2 BONCAT-FCM compared with ^{3}H-Leucine

Samples on the CCE-LTER P2107 process cruise were collected using the BONCAT-FCM and ^{3}H-Leucine protocol described. A total of 210 samples were taken using each protocol from the three sampling cycles. For surface water samples, bacterial production determined by ^{3}H-

Leucine radioisotopic labelling and bacterial activity determined by BONCAT bulk fluorescence were compared (Figure 2.3). There is some agreement between the two methods with bacterial production and bacterial activity being highest in sampling cycles 1 and 2. These cycles correspond to the highest primary production represented by satellite chlorophyll *a* measurements. Sampling cycle 3 had the lowest amount of measured bacterial activity from both methods. Sampling cycle 3 was conducted in open ocean oligotrophic waters with low

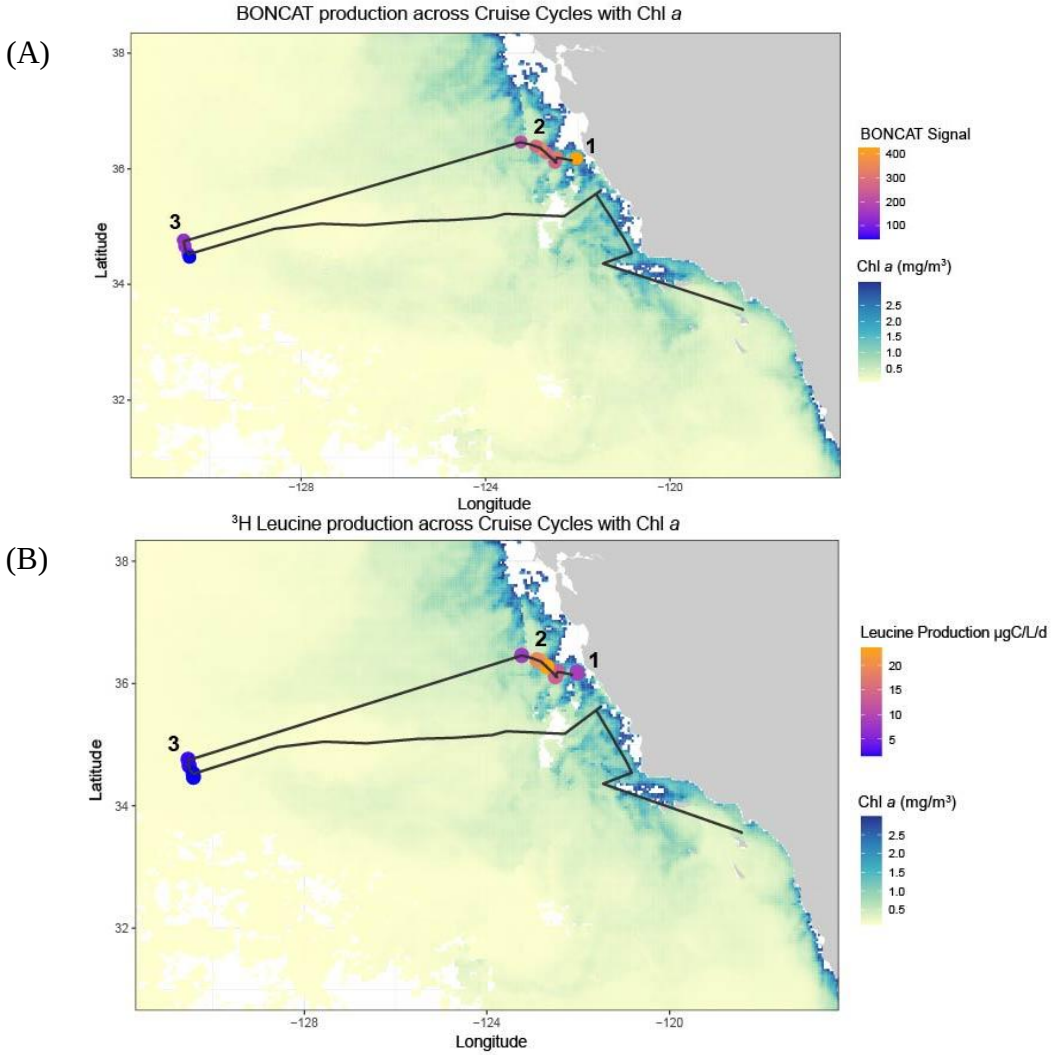


Figure 2.3: Chlorophyll *a* maps with BONCAT (A) and 3H-Leucine production (B) values for upper depths from the three CCE P2107 sampling cycles. The cruise track is shown in black with numbers corresponding to the location of the sampling cycles. Chlorophyll *a* values mapped are averages across sampling dates. BONCAT values are expressed as Median Fluorescence Intensity (MFI) obtained from flow cytometry data.

Chlorophyll *a* measurements.

The results from the ^{3}H-Leucine radioisotopic labelling were compared directly to the BONCAT-FCM bulk fluorescence results with linear regression to test the agreement between the methods (Figure 2.4). Both sets of measurements were log transformed prior to the regression analysis. The BONCAT-FCM bulk fluorescence measurements had a weak (R^2=0.25) but significant relationship with the ^{3}H-Leucine results. An overall positive trend was present in the data. Both methods had higher estimates at depths closer to the surface than at deeper depths.

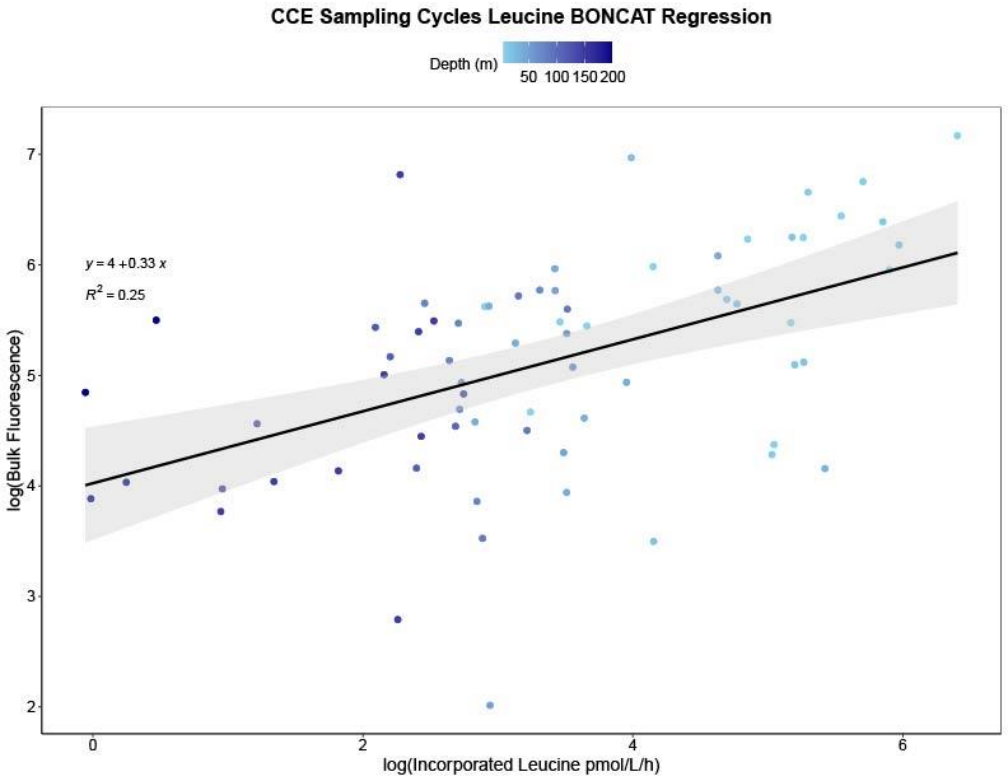


Figure 2.4: Scatter plot of bulk fluorescence calculated from CCE P2107 BONCAT-FCM samples. Fitted regression line is plotted in black with 95% confidence intervals in grey. Scatter points are colored by the depth of sample collection. Line equation and R^2 displayed on plot.

The percentage of active cells was obtained by taking the proportion of active cells determined by FCM analysis from the total amount of cells identified from the SYBR Green DNA staining. This percentage of active cells differed with depth and across different sampling days. The active percentage was compared across sampling cycle 2 which had the highest

amount of estimated BP. The identified active percent was generally lower at deeper depths and higher near the surface waters or the subsurface chlorophyll maximum (Figure 2.5). There was a degree of variability among the triplicate samples for each depth.

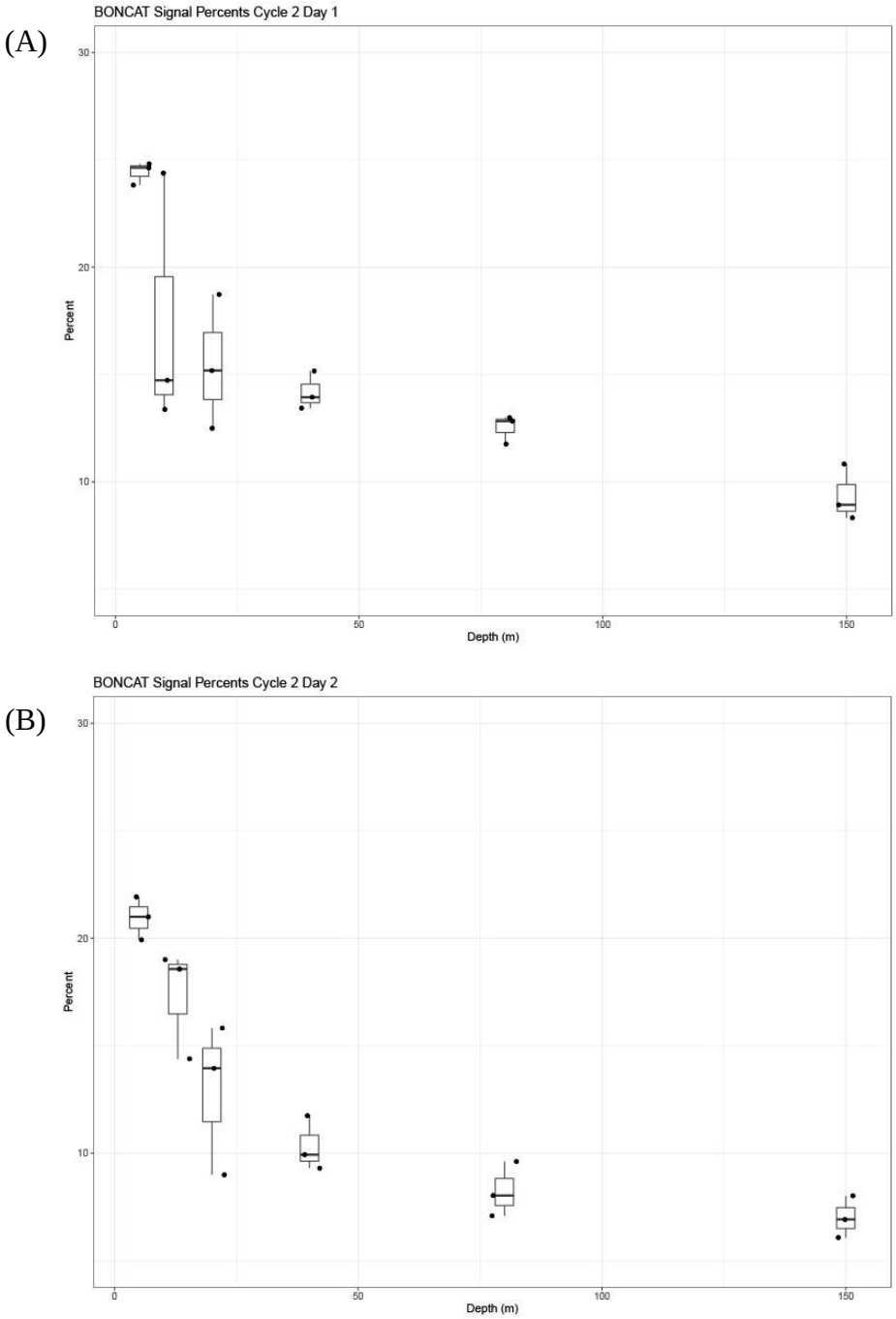
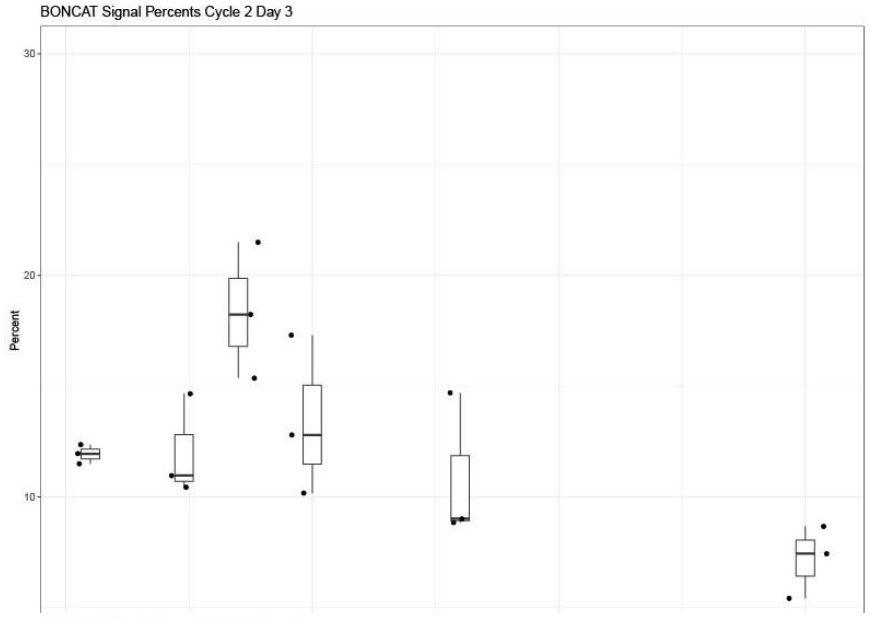


Figure 2.5: Boxplots of active cells identified using BONCAT-FCM methods across all sampling depths from sampling cycle 2 of the CCE-LTER P2107 process cruise. Day 1 (A) and Day 2 (B) had the highest active percent while Day 3 (C) and Day 4 (D) were more variable. Scatter points shown represent individual sample values since samples were taken in triplicate at each depth.

(C)



(D)

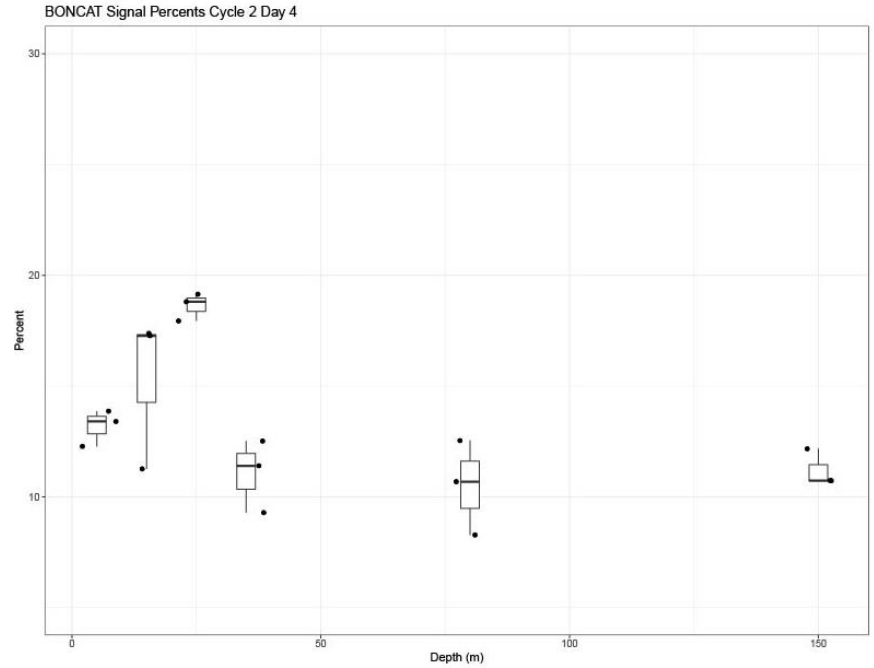


Figure 2.5: Boxplots of active cells identified using BONCAT-FCM methods across all sampling depths from sampling cycle 2 of the CCE-LTER P2107 process cruise, Continued. Day 1 (A) and Day 2 (B) had the highest active percent while Day 3 (C) and Day 4 (D) were more variable. Scatter points shown represent individual sample values since samples were taken in triplicate at each depth.

2.4 Discussion

Much attention has been devoted to developing new methods for measuring marine microbial activity to obtain a better understanding of the global oceanic ecosystem. A variety of new methods have emerged for measuring the activity of marine bacterial communities with biorthogonal noncanonical amino acid tagging (BONCAT) gaining notoriety as an easy and low-cost technique (Hatzenpichler et al., 2014). The BONCAT-FCM work here further highlights the potential of this technique in studying the active portion of the marine microbiome. This BONCAT-FCM protocol was compared to standard ^{3}H-Leucine radioisotopic labelling on the CCE P2107 process cruise to compare and validate the bulk fluorescence quantification as an estimate for BP. While the BONCAT-FCM method identified some similar trends in BP measured from the ^{3}H-Leucine method, a direct comparison of samples using regression analysis showed only a weak relationship between both methods. There were some sampling days that had greater differences in results between the two methods. BONCAT bulk fluorescence signal was consistently higher than the corresponding ^{3}H-Leucine incorporation on the first day of sampling cycle one but lower on the first day of sampling cycle 2. Due to limited time and resources, the BONCAT-FCM protocol was not compared and optimized alongside the ^{3}H-Leucine protocol in a controlled laboratory setting. It's likely that the BONCAT-FCM method would more closely correlate with the ^{3}H-Leucine method with further testing in a more controlled environment as done in previous studies using HPG and CuAAC (Leizeaga et al., 2017; Samo et al., 2014). Additionally, the described BONCAT-FCM method here was developed and tested on surface water samples (above 10 meters). Since the bacterial community structure can change considerably throughout the water column, the BONCAT-FCM method might not fully capture the BP of deeper bacterial communities (Cram et al., 2015). The

incubation conditions on the research vessel could also have affected the efficiency of the BONCAT-FCM method. The effect of not incubating environmental samples at in-situ pressure and temperature conditions has been known to result in less active microbial communities and underestimated BP measurements using ^{3}H-Leucine (C. Tamburini et al., 2013). It is currently unknown how these incubation conditions could affect the efficiency of BONCAT-FCM.

The described BONCAT-FCM protocol using AHA and SPAAC labelling identified up to about 30% of the total identified marine bacterial population in the gated region representing BONCAT signal. The identified percent of the active population did change with depth and among the sampling cycles. The lowest active percentages were found at the deepest depths, where BP was seen to be lowest from ^{3}H-Leucine radioisotopic labelling as well. This agreement suggests that the BONCAT-FCM method is effective at identifying some portion of the active bacterial population. In culture studies using BONCAT, the active percentages generally reached above 90% but a short incubation time was used here to reduce the risk of biochemical disturbances in cellular function from prolonged incubation (Hatzenpichler et al., 2014). A shortened incubation time also helps minimize the effect that the lack of in-situ conditions could have on the translational activity of the cells (Sherr et al., 1999). There was a notable percentage of background fluorescent events in the killed controls, sometimes equaling that off the BONCAT samples. One possible reason for the observed background fluorescence could stem from the non-specific binding in the SPAAC protocol. DBCO dyes are known to non-specifically bind to cysteine residues resulting in higher background fluorescence (E. J. Kim et al., 2013). While pre-incubation with a haloacetamide such as 2-chloroacetamide helps limit the amount of non-specific binding by alkylating cysteine residues, nonspecific binding could still occur (van Geel et al., 2012). The treatment of samples prior to the SPAAC protocol might also affect the

results. Samples were fixed by adding formaldehyde to a final concentration of 4% for two hours. Formaldehyde was chosen as the fixative due to its use with marine samples and the lower degree of inter-cellular cross-linkage compared to glutaraldehyde (Kamiya et al., 2007; Kiernan, 2000). Increased cross-linkage could reduce the efficiency of the washing steps for removing unbound AHA and fluorescent dye. However, Lindivat et al. (2021) found that killed control samples fixed with formaldehyde and subsequently incubated with HPG exhibited fluorescent populations from FCM analysis similar to BONCAT samples. The scenario that excess AHA is trapped in fixed cell membrane may thus be likely. Reducing the concentration of AHA could possibly reduce the amount of free AHA available to become trapped in the fixed bacterial cells.

Furthermore, the gates used in FCM analysis were applied manually to capture the most fluorescent events separated from the main population. Several improvements to the gating method would enhance the capture of the active portion of the environmental bacteria samples. The use of automated gating algorithms using self-organizing maps or other methods could considerably increase the accuracy and efficiency of processing batches of samples (H. Lee et al., 2019). The strain-promoted click chemistry step is crucial in successfully visualizing and identifying the active labelled portion of the microbial community using FCM analysis. The DBCO dye used in this study is excited by the violet laser (405 nm) on the flow cytometry and the signal is detected in the blue-violet channel. The fluorescence of this dye is weaker than other commercially available dyes which could explain the lack of definition in the sorted active population (Lindivat et al., 2020). Lindivat et al., (2020) found that labelling dyes that are excited by longer wavelength lasers, like AZDye 647, resulted in a more distinct population of fluorescent events. The Guava Easycyte flow cytometer used in this study is only equipped with one violet and one blue excitation laser. Since samples are counterstained with SYBR green

DNA stain that is excited by the blue laser, the spectral range of the dye for SPAAC labelling was limited to the violet laser wavelength to prevent fluorescence overlap.

With the relatively new application of BONCAT methodology to marine environments there is still much to learn to better develop this technique. Currently, the mechanism for how the synthetic amino acids AHA or HPG enter the cell is unknown (Hatzenpichler et al., 2020). BONCAT is reliant on the incorporation of AHA into proteins by binding to inter-cellular methionyl-tRNA synthetases. The differing amount of methionyl-tRNA synthetases among a bacterial consortium would result in differences in signal among species in a community (Hatzenpichler et al., 2020). Further, the aminoacylation of AHA and HPG has been found to differ in pure *Escherichia coli* cultures, suggesting possible differences in use between the two synthetic amino acids (Beatty & Tirrell, 2008). While the use of synthetic amino acids like AHA and HPG has been thought to not significantly alter the physiology or metabolomics of cells, there is some evidence that changes do occur. Steward, et al. (2020) found that HPG and AHA incubation did alter the metabolic profiles of *E. coli* cultures with HPG causing a more profound effect under heat stressed conditions though the observed alterations were not drastic. Previous studies with *E. coli* also showed that long-term incubations with AHA arrested growth in pure culture (Kramer et al., 2009). It is currently unknown if stress from decompression of environmental samples corresponds with physiological perturbations from synthetic amino acid incubation. Studies with plant seedlings found that incubation with AHA resulted in stunted growth while incubation with HPG had less of an effect (Tivendale et al., 2021). In the cyanobacterium, *Synechococcus*, HPG incubation has been shown to negatively affect growth and even crash cultures under higher concentrations, though the presence of this effect differed among algal species (Michels et al., 2021). The concentration of AHA used in this BONCAT-

FCM protocol may thus be negatively affecting certain bacterial species and not capturing the entire active population from environmental samples. One possible reason for the differences between AHA and HPG could be caused by the differences in methionyl-tRNA synthetases binding affinity of the two synthetic amino acids (Kiick et al., 2002; van Hest et al., 2000). The higher binding of AHA would result in more protein incorporations which could result in faster degradation of downstream cellular functions. The current research does highlight some questions about the scope of application of BONCAT techniques. With observed effects from BONCAT methods differing depending on species and synthetic amino acid used, more research is needed to understand the possible detrimental effects.

Much of the current application of BONCAT in marine environments has been done using HPG and copper catalyzed click chemistry. One reason for the use of HPG is due to the stability issues of AHA under conditions of high pH and high sulfide concentrations found in environments like marine sediments (Hatzenpichler et al., 2014). As previously stated, there are differences between the use of HPG and AHA and their effects on bacterial physiology and metabolomics that need further investigation. The benefit of this BONCAT-FCM method with AHA opposed to HPG is the compatibility with the rapid and easy SPAAC protocol with DBCO-bound dyes. The PPMA beads used for the positive controls also were only available with azide functional groups at the time this protocol was developed. Since then, DiaPoly Alkyne beads have been manufactured and can be used to establish positive controls for the CuAAC protocol. SPAAC also has the benefit to be used in-vivo as the essential reagents are not toxic to cells (Bergkessel & Delavaine, 2021). However, recent breakthroughs in copper catalyzed click chemistry by using picolyl azide dyes resulted in faster reactions time using less Cu concentration while significantly increasing the signal distinction (Uttamapinant et al., 2012).

With less studies using BONCAT with AHA and SPAAC in marine environments, a direct comparison with BONCAT using HPG and CuAAC is necessary to highlight any differences between the two methods.

With the advances in genomic technology over the past decades, the lens to study marine bacterial communities has magnified considerably. The use of metagenomics and other genetic techniques has spurred a deepening of the understanding of bacterial diversity and abundance as well as function in the ocean (H. H. Kim et al., 2022; Sunagawa et al., 2020; Venter et al., 2004). This use of metagenomics provides a truly unprecedented amount of information about the environmental prokaryotic community structure and function. As genomic technology continues to advance it is important that techniques to visualize bacterial species activity advance as well. With the difficulty in modeling bacterial production and ecosystem function from genomic analysis alone, the added information from visualization techniques like BONCAT might be crucial (Sebastián & Gasol, 2019). The versatility of BONCAT methods can even be further combined with many downstream processing applications to obtain useful insights. Using fluorescence activated cell sorting, the fluorescently labelled active portion of a sample can be sorted out and sequenced to identify the translationally active species of a sample (Chen et al., 2021; Couradeau et al., 2019; Du & Behrens, 2021; Hatzenpichler et al., 2016; Reichart et al., 2020). BONCAT coupled with fluorescence in-situ hybridization (BONCAT-FISH) has also been successfully used to visualize and study the ecophysiological activity of marine bacteria and archea (Hatzenpichler et al., 2016; Pereira et al., 2022). Moving forward, the scope of environmental microbiological studies can be broadened further by using BONCAT techniques in conjugation with stable-isotope probing (SIP) or secondary-ion mass spectrometry (NANO-SIMS) to characterize the microbial activity and influence on biogeochemical cycling of

microbial populations in an environment (Marlow et al., 2021; Pasulka et al., 2018; Thangavelu et al., 2021).

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