***Original Research Article***

**Estimation of Microbial Abundances and Their Respiration Rates in a Specialized Coastal Water Body**

**ABSTRACT**

Microbial communities in an ecosystem are crucial for having active participation in shaping the biological and biochemical cycles of this ecosystem. In this study, we estimated marine bacterial counts and the microbial respiration rates in seawater samples collected from Gorliz beach, Bilbao, Spain, using DAPI-based epifluorescence microscopy and optical oxygen sensors. The microbial density was 1.6 × 10⁶ cells/mL (± 47,948 SE), which seemed to be consistent with typical values for temperate coastal regions. In addition, microbial respiration rate based on the oxygen consumption was around a mean of 0.0127 μmol O₂/L/h (± 0.0026 SE) over 14 hours of incubation in the dark. These findings suggested an abundant and meaningful active microbial community in this coastal ecosystem and emphasized the significance of microorganisms in shaping coastal ecosystem dynamics, local oxygen budget, and carbon biogeo-cycling. Therefore, the study would underline the need for sequential, spatial, and temporal investigations of microbial communities and their biochemical processes in response to environmental changes and anthropogenic activities.

*Keywords: Microbial communities, oxygen consumption, coastal ecosystem, temperate region.*

**1. INTRODUCTION**

The marine entity is considered the world’s largest ecosystem, covering more than two-thirds of the Earth’s surface, which includes multivarious marine life and their communities owing to a series of environmental situations such as polar and tropical waters, sub-seafloor and sunlit surface, open and coastal waters, etc. All these habitats contain marine microbes, such as bacteria, viruses, protists, and fungi and these are one of the most important and diverse communities in the marine ecosystem. Marine microbes encompass the three realms of life, like Bacteria, Archaea and Eukarya, along with viruses and viroids (Bolhuis and Cretoiu, 2016; Massana and Logares, 2013). They are actively involved in marine biogeochemical cycles, food webs, and thereby in the ecosystem, playing an indispensable role in regulating the marine environment. Marine microbes are the prominent digesters of oceanic organic matter, which denotes a substantial biomass production in saline ecosystems in the case of microorganisms. A recent review says, the relative contribution of microbes to carbon fixation and nitrogen cycle is huge in oligotrophic and ocean ecosystems. In contrast, bacterial and microbial respiration accounts for the largest part of oxygen consumption in the seawater environment and thus, leading to oxygen minimum zones (OMZ) and hypoxic zones in the ocean (Diaz and Rosenberg, 2008; del Giorgio and Williams, 2005). Therefore, proper estimation and counting of bacterial density, abundance and composition are of crucial need to determine microbial population dynamics and their role in marine environments. Measuring bacterial respiration rates is also important for observing bacterial growth efficiency (BGE), abundance, and understanding the ratio of carbon between the production of biomass and CO2 discharge. A better insight into bacterial respiration rates and their regulation on the growth curve would help to explain the ocean carbon cycles, hypoxic zones in the ocean and properly manage the marine environment.

Advances on exploring marine microbiomes and their ecological roles are gaining concern during the recent years (e.g., Fuhrman, et al., 2015; Giovannoni and Vergin, 2012; Yilmaz et al., 2016; Zhang et al., 2015). Yet, understanding the roles and functioning of microbes in marine ecosystems is still lacking. Predominantly, the impact of global changes on the marine microorganisms is largely unknown. Recently, some studies on marine microbes and their ecosystems have been carried out beyond microbial habitats with special focuses on archaeal and viral communities (e.g., Gustavsen et al., 2014; Hugoni et al., 2013; Sintes et al., 2015; Yilmaz et al., 2016; Zhang et al., 2015). Particularly, the ecological role of the coastal microorganisms in coastal ecosystem functioning and services demands detailed and continuous investigation, particularly since the coastal zone is continuously impacted by anthropogenic interferences and subjected to significant environmental differences, including freshwater runoff, river discharge and effluent from wastewater treatment, etc.

The Bay of Biscay, derived from the Atlantic Ocean, displays a thermal stratum and occasional coastal upwelling events (Botas et al. 1990). These coastal phenomena are created due to the prevailing winds, which are northeastern in summer seasons and build westward superficial currents, constructing an Ekman transport offshore (Botas et al. 1990). These activities make the Bay of Biscay more dynamic and rapidly changing in its faunal communities, especially microbial communities. In addition, the Bay of Biscay, being much larger, has many scattered coasts along the bank of this Bay, which differs the bacterial abundance and respiration from one beach to another. Gorliz beach, Plentzia at the bank of the Bay of Biscay (Bilbao, Spain) is one of them, which has a riverine flow and is a little bit distracted from the main flow of the Bay of Biscay. Although some previous studies in the Bay of Biscay have shown some microbial profiles and their ecological role (Marañón and Fernández 1995; Bode and Fernández 1992; Barquero et al. 1998), there is no such study on marine microbes at this part (Gorliz beach point) of the Bay of Biscay. Therefore, this study aimed to estimate the microbial abundance and respiration rates in the seawater sample of this beach point. In this study, we measured bacterial counts and respiration rates in the seawater sample collected from the Goliz beach.

**2. MATERIAL AND METHODS**

**2.1 Study area and sample collection**

Gorliz, Plentzia (around 43.41°N and 2.95°W) is a coastal area, located in the province of Biscay, Spain, which is along the south-eastern edge of the Bay of Biscay (a part of the north-eastern Atlantic Ocean) (**Figure 1**). The area is familiar for its mixed hydrodynamics and richer biodiversity, holding a variety of coastal habitats and communities and influenced by Atlantic swells, seasonal upwelling, and riverine input. To some extent, Gorliz is represented as a model study site for temperate marine ecosystem.

For this experiment, seawater sample was collected on 27 April 2023, from the mentioned study area near the PiE-UPV/EHU station. The sample type was kind of low low-sediment biofilm seawater, which was collected 10 meters away from the breaking waves. Around 1 liter of seawater sample was collected in a glass jar, and from this sample, two subsamples of 10 mL were drawn into 20 mL plastic bottles using a micropipette. Then it was homogenized by mixing and kept in a dark place in the laboratory.

**2.2 Estimation of Microbial Abundance**

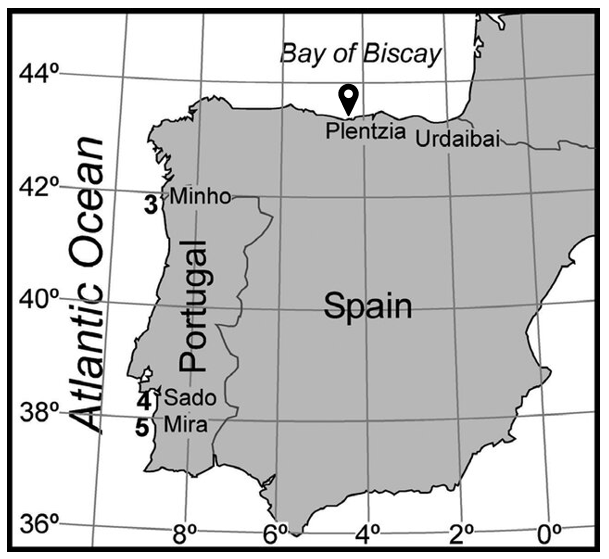
In this experiment for counting marine microbial abundance in a seawater sample, we used the Epifluorescence microscopy and DAPI (4 ',6-diamidino-2-fenilindol) as a staining fluorochrome. To perform this experiment, we followed a modified protocol taken from Lebaron et al. (1998) and Noble and Fuhrman (1998). At first, we collected 1 L seawater sample from the Gorliz beach and stored it in a cool place in the dark. Two 10 mL subsamples were stained and filtered immediately. Then, subsamples were fixed with 0.2 μm-filtered borax-buffered formalin (2% v/v final concentration) and incubated on ice for 10 minutes. Later, from each subsample, we took 1 mL volume in the two-mL clean and sterilized micro-centrifuge tubes and added 70 μL of the DAPI solution to each tube (2 μg mL-1 final concentration) for staining. We mixed them thoroughly and incubated on ice in the dark for 15 minutes to facilitate effective DNA binding.

After that, for filtration the sample, a 0.2 μm pore size black polycarbonate filter mounted over a Whatman cellulose pre-filter was used. When filtration was done, we collected the filter carefully with a clean forceps onto a prepared glass microscopic slide. Microscopic observation was done through a UV-excitation filter set and a 100× oil immersion objective. We counted the cell counts, maintaining randomly selected fields, around ≤30 cells per field, and the field scaling factor was 30,000 fields/filter. The counts can be used to compute the number of cells per milliliter of saltwater. Finally total estimation of bacterial counts was calculated using a specific formula with statistical analysis (**Table 1**).

**2.3 Estimation of Microbial Respiration Rates**

In this part, to estimate the respiration rates of microbes present in the samples, the sample was filtered through a 100 μm mesh before measuring the microbial respiration rate. After that, we divided the samples into four oxvials carefully if there are no bubbles and then stored in the same rack. All the samples from each group are connected with a single firesting system. Each oxvial has a specific sensor spot, which is in touch with the sample. One vial is filled completely equipped with the "Redflash" sensing strip with the sample, avoiding the formation of bubbles in the process and a fifth plastic vial was used to insert the temperature probe. From the optical, a red light is emitted which depends on the concentration of oxygen in the oxvial and it excites the sensor at an NIR wavelength. So, there is an inverse relationship between oxygen concentration and NIR excitation. The NIR is transferred to the firefighting system, in which it is digitized and interpreted on the oxygen logger software. According to the default system, each measurement is taken every minute for 24 hours, and the data is produced in text format.

Oxygen consumption was measured from the regression lines of oxygen concentration against incubation time, ranging from 3–8 hours, where the decreasing line was highly linear and stable. Final respiration rates were calculated taking into account the mean of control vials and expressed as μmol O2/L/h (**Table 2**). Data were analyzed and plotted using Microsoft Excel.

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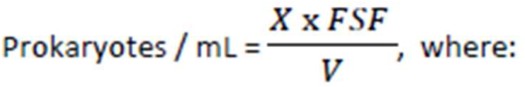
**Fig. 1. The map shows the study area, Gorliz, Bilbao, Spain** (Modified from Leorri et al., 2010).

**Table 1. Overview of the calculation for the microbial counts**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  | **Counts**  **(Replica 1)** | **Counts**  **(Replica 2)** | **Counts**  **(Replica 3)** | **Final estimation** | |
|  | 30 | 10 | 25 | N | 3 |
|  | 60 | 10 | 31 | Mean (M) | 1603000 |
|  | 52 | 11 | 33 | Variance | 6897000000 |
|  | 55 | 12 | 28 | Standard deviation (SD) | 83048.17879 |
|  | 61 | 8 | 34 | Standard error (SE) | 47947.88838 |
|  | 50 | 9 | 25 | Coefficient of variation | 5.18079718 |
|  | 61 | 12 | 20 |  |  |
|  | 62 | 9 | 30 |  |  |
|  | 42 | 11 | 27 |  |  |
|  | 65 | 9 | 32 |  |  |
| Average | 53.8 | 10.1 | 28.5 |  |  |
| Filtered volume | 1mL | 0.2 | 0.5 |  |  |
| Prokaryotes/mL | 1614000 | 1515000 | 1680000 |  |  |
| Final result: M(±SE) = 1603000 total prokaryotes/mL (±47947.88838) | | | | | |

The calculations leading to a final estimation of bacteria abundance in the sample

For, ≤30 cells/ﬁeld :



X = the average number of prokaryotes counted per ﬁeld

FSF= the ﬁeld scaling factor = 30000 fields/filter

V = the volume of seawater ﬁltered (mL)

**Table 2. Overview of the calculations for the estimation of respiration rates**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Replicate** | **Respiration rate**  **μmol O2/L h** | **Respiration rate corrected**  **μmol O2/L h** | **Final respiration rate corrected** | |
| 1 | 0.0247 | 0.01255 | N | 3 |
| 2 | 0.0294 | 0.01725 | Mean (M) | 0.012683333 |
| 3 | 0.0204 | 0.00825 | Variance | 2.02633E-05 |
| control 1 | 0.0179 |  | Standard deviation (SD) | 0.004501481 |
| control 2 | 0.0064 |  | Standard error (SE) | 0.002598931 |
| Average control | 0.01215 |  | Coefficient of variation | 35.4913107 |
| Final result: | M(±SE)= 0.012683333 μmol O2/L h (± 0.002598931) | | | |

**3. RESULTS AND DISCUSSIONS**

The microbiome tells a lot about its surrounding environment and ecology, offering a key insight as an indicator of the ecosystem. The study on the estimation of microbial availability, density, and their respiration demand in the aquatic environment of Gorliz Beach at the Bay of Biscay revealed some baseline insights on the microbial activity and their ecological abundance in a temperate Atlantic environment. In this experiment, we estimated the abundance and respiration rates of microorganisms through epifluorescence microscopy, DAPI, and optical oxygen sensors over the period of 14 hours of incubation without light.

Microscopic counts with DAPI staining confirmed an average microbial density of 1.6 × 10⁶ cells/mL (± 47,948 SE) (Table 1). Here, we found the cell counts consistent in all the fields of all the replicates, taking a microscope scaling factor (30,000 fields/filter) and a volume of 1 mL (filtered). The coefficient of variation for microbial counts was 5.18%, which was comparatively low and indicates evenly distributed microbes in the samples. After filtering and fixing, the subsamples contained enlightened DAPI-stained cells.

Our findings, the count we got (1.6 × 10⁶ cells/m), were found consistent with the typical values of microbial counts documented in coastal areas with moderate nutrients. The high load of microbial communities indicates a dynamic and suitable habitat for microbes regulated by hydrodynamic activities, land overflow, precipitation, and mildly occasional upwelling characteristics of this southeastern part of the Bay of Biscay (Botas et al., 1990; Thomson et al., 2010). Moreover, the significant microbial presence found defines the Gorliz beach as an active biological coastal entity because such microbial loads are observed in near-shore marine areas, where nutrient inflows are sourced from river flow and humanized sources like wastewater discharge, promoting microbial abundance (Dai et al., 2022).

On the other hand, microbial respiration rates were examined by observing the consumption of oxygen in dark incubation over 14 hours. We observed a visible and linear decline in oxygen concentrations in all replicates (Triplicates). At this time, the control samples exhibited negligible changes in oxygen concentration (**Figure 2**). In this figure, a distinct difference was observed between the trends of experimental samples and control samples. The oxygen concentration in the experimental samples (Ch1, Ch2, Ch3) decreased consistently over the incubation period, where sample Ch2 showed the sharpest fall, indicating high microbial loads and communities. Contrary, we found stability in all control samples (Control Ch1 and Ch3) treated by sterilization, which confirmed that the oxygen decline measured in the experimental (untreated) samples was due to biological activities (respiration) of the microbes.

There was a huge difference in temperature evolution between the samples and controls during the incubation time. For the experimental samples, an average temperature was approximately 21°C, which remained almost constant over the whole incubation time (**Figure 3**). On the other hand, the control vials, which went through boiling during sterilization, produced higher temperatures (~43°C), further ensuring the authentication of inactivation treatment. This consistency in temperature in the treatment samples ensured that oxygen consumption was due to microbial respiration and metabolic activities rather than thermal fluctuation.

Finally, for the accurate measurement of the respiration rate, we used only the maximum linear regression of the oxygen depletion curve (between 3 and 8 hours) (**Figure 4**). The figure, showing the regression lines for both sample and control vials, reveals a steeper and negative slope for all the experimental samples:

Ch1: –0.0247 μmol O₂/L/h

Ch2: –0.0294 μmol O₂/L/h

Ch3: –0.0204 μmol O₂/L/h

Contrary, the controls the slopes were:

Control Ch3: –0.0179 μmol O₂/L/h

Control Ch1: –0.0064 μmol O₂/L/h

The regression lines indicate substantial oxygen consumption by the microbial community. After correcting background oxygen taken in controls, we found the final mean respiration rate of microorganisms 0.0127 μmol O₂/L/h (± 0.0026 SE), which reflected the metabolic activities of the microbial community in this coastal area. Although among the replicas, there was some disparity (coefficient of variation ~35.5%), the trend was found to be consistent and biologically interpretable in all the samples.

Therefore, altogether, the microbial density and oxygen consumption rate confirmed that the Gorliz Beach is rich in microbial communities and their biological activities. The rate we observed for the respiration aligned with previous data for temperate coastal areas and highlighted the role of microbial communities in recycling organic stocks and oxygen turnover (del Giorgio and Williams, 2005). The measured moderate oxygen level can support substantial microbial activities, which significantly impact local oxygen demands in coastal areas. However, under stratification or eutrophication, it may possibly stimulate the creation of hypoxia (Diaz and Rosenberg, 2008).

Notably, in combination of microbial load and their respiration rates (oxygen consumption) provides an estimation of biological activities of microorganisms. Considering an even participation from all prokaryotic cells, the mean respiration rates of all the cells support the area as oligotrophic to mesotrophic waters (Diaz and Rosenberg, 2008). This stability between cellular abundance and their metabolic activities is considered a vital indicator in understanding microbial carbon turnover and the functionality of the ecosystem. The oxygen consumption found displays representativeness of heterotrophic activities, which could support the statement that coastal microbial communities have a significant role in degrading organic matter and in regulating oxygen turnover, mostly under stratification, upwelling, or heavy nutrient situations (Diaz and Rosenberg, 2014).

In summary, this study provides preliminary information on microbial abundance and their ecological functioning in a coastal ecosystem of a temperate region such as the Bay of Biscay. This simultaneous study on measuring microbial abundance and respiration activities hopefully encourages the need for a detailed study in a robust way with multiple temporal and spatial considerations to assess microbial diversity and their ecological functions in the coastal ecosystem, as coastal areas remain saturated with anthropogenic pressures, inland flow, and tourism activities. Therefore, regular assessments and detailed studies of microbial density and diversity could potentially offer a biological indicator of ecosystem soundness. Future and further investigations into exploring community structure and metabolic activities (e.g., via molecular 16S rRNA sequencing, metagenomics, and transcriptomic profiling) with a robust dataset would offer a deeper insight into the ecological significance and ecosystem responses of coastal microorganisms under different environmental changes.

**Fig. 2. Temperature evolution of sample (Ch2) and control (C) over the incubation period of 14 hours showed that the experimental samples became stable at 21°C, approximately, and the elevated control ones reflected heat sterilization.**

**Fig. 3. The combined figure showed the oxygen concentration (μmol O2/L) in 3 experimental samples (Ch1, Ch2, Ch3) and 2 controls (Ch1, Ch3) oxvials along with the evolution the temperature during a 14-hour dark incubation. The blue line indicates the time when the values (temperature and oxygen concentration) were relatively found to be stable and considerable for counting the concentration.**

**Fig. 4. Oxygen consumption between 3-8 hours. Linear regression of oxygen concentration showed a continuous decline between 3 to 8 hours in treatment and control samples. Slopes were significantly steeper in triplicate (Ch1: –0.0247, Ch2: –0.0294, Ch3: –0.0204 μmol O2/L/h) over the control samples were significantly steeper than controls.**

**4. CONCLUSION**

The study estimated microbial density and their metabolic rate (oxygen consumption) in the seawater of Gorliz Beach (Bilbao, Spain), a curved extension derived from the Bay of Biscay in the Atlantic Ocean, regulated by both natural and anthropogenic parameters. The result significantly indicated the presence of microbes and their active participation in biological dynamics in this partly separated coast of the Bay of Biscay. While the experiential findings are aligned with these types of environments, however, their effect may be weighty under stratification or eutrophication, potentially in hypoxia or dead zones in any coastal ecosystems. More studies are being suggested to be conducted in a temporal and spatial basis, along with molecular approaches to deepen the understanding of microbial abundance and their biological activities, and ecological dynamics in response to environmental changes.

**DISCLAIMER (ARTIFICIAL INTELLIGENCE)**

Authors hereby declare that NO AI generative technologies such as ChatGPT, COPILOT, and text-to-image generators have been applied during the writing or editing of manuscripts.

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