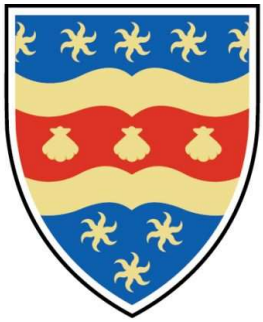
REDUCED ERGOSTEROL AND FATTY ACID METHYL ESTHERS' COMPOSITION OF MARINE ANOXIC FUNGI



UNIVERSITY OF PLYMOUTH

Kalina Karadimova

10469804

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Project Advisors: Michael Cunliffe, School of Biological and Marine Sciences, Emily Cooper, School of Geography, Earth and Environmental Sciences,

University of Plymouth, Drake Circus, Plymouth, PL4 8AA

Fungal Ecology

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Kalina Karadimova

Project Advisors: Emily Cooper, Michael Cunliffe

Abstract: Marine Fungi are ubiquitous, yet severely understudied, with papers on anaerobic fungi found in anoxic zones lacking. In this study, we compared profiles from aerobically and anaerobically grown *Mucor carcinelloides* and found significant differences that were directly related to oxygen presence. Both saturated and unsaturated fatty acid methyl esters (FAMEs) and ergosterol decreased by five times when deprived of oxygen. These results are important since ergosterol and 18:2w6 are both labelled biomarkers for fungi, yet both were seen less in the anoxic conditions. Given their requirement of molecular oxygen for synthesis such observations were expected; yet it implied the possible unsuitability of ergosterol as a biomarker. The presence of ergosterol and unsaturated FAMEs in the no oxygen conditions, including the odd-chain pentadecanoate, indicated the possibility of an alternative anaerobic metabolism or cell adaptation and evolution.

Keywords: marine fungi, ergosterol, FAMEs, anoxic, oxic, biomass

Introduction

Fungi are highly adaptable, and therefore, can grow well under a range of freshwater and saline conditions. They are widely distributed in marine environments from polar ice covers to the deep sea (Nagano *et al.*, 2010; Imhoff, 2016), and are found in nearly all explored marine habitats (Amend *et al*., 2019; Gladfelter *et al.*, 2019). They are present in marine sediments, brackish waters, marine algae, intertidal pools, sea fans, sponges (Amend *et al*., 2019; Gladfelter *et al.*, 2019), and rhizosphere of mangroves (Gupta *et al*., 2007; Oeskser, 2014; Imhoff, 2016). Some are even theorized to be amphibious given their correlation with abiotic environmental conditions and gene expression data (Amend *et al*., 2019). Marine fungi are a fungus found in marine habitats that can form symbiotic relationships, has evolved/adapted and continued to do so at a genetic level, and can grow, and reproduce in marine environments. (Pang *et al*., 2016; Amend *et al*., 2019). By 2002 most fungi were named obligate (Zhoul *et a*l., 2002). As late as 2010, the ecology and roles of fungi in deep-sea environments were still unknown; Surprising, given the fact, fungi are found at the deepest place known to humans – the Marianna Trench. They show no significant difference with

diversity, meaning depth does not necessarily equal more diversity (Nagano, *et al*, 2010). Yet, a paper two years later found fungal signatures in the Central Indian Basin had greater diversity in deep sea sediments (Singh *et al*., 2012). Some are detected at oxygen-deprived deep see sites only (DSF-Group1) such as below the sea floor, methane cold-seep, or bacterial mat- proof of anaerobic fungi. (Nagano *et al*., 2010). Others were found to have been buried for 30,000 years at depths of 30cm below sea floor (Singh *et al*., 2012).

Their roles include saprotrophs on detrital organic matter (aquatic and terrestrial); (Gladfelter *et al*., 2019), decomposing cellulose and chitin (Kohlmeyer and Kohlmeyer, 1979), contributing to living microbial carbon (Amend *et al*., 2019), a potential "fuel" for trophic webs in the ocean (Guiterezz *et al*., 2019) and marine sediment chemistry (Amend *et al*., 2019). Fungi play a critical role even in freshwater systems. They infect phytoplankton, making it easier pray to zooplankton thus influencing the system nutrient dynamics (Amend *et al*., 2019). Affecting fungal abundance, fungi have an impact on *Symbiodinium*, a dinoflagellate symbiont. When they bloom in coastal waters, nonchytris fungi elicit a defensive response in corals (Amend *et al*., 2019) or even, pathogens of organisms in the deep sea- *Sagenomella* (Singh *et al*., 2012). Some Parasitic fungi positively impact zooplankton's diet via their action on diatoms in freshwater ecosystems (Kagami *et al*., 2011; Gladfelter *et al*., 2019). That happens when inedible phytoplankton gets infected by chytrids, and nutritive-rich flagellated zoospores are released (Kagami *et al.,* 2011). Their role as organic detritus modifiers in some coastal environments has been widely recognized (Guiterrez *et al*., 2019). Therefore, these various statements bring the theory that fungi can be a source of otherwise inaccessible energy for marine trophic webs. The same paper also discusses that the carbon and nitrogen ratios of organic matter can be impacted by the fungi's activity and occurrence. This can potentially lead to the source of organic matter in detrital and sedimentary environments being misinterpreted. Thus, it can be concluded for the moment that marine fungi's role in carbon and nutrient cycling in the ocean (Valiela, 2016; Amend *et al*., 2019; Gladfelter *et al*., 2019; Grossart *et al*., 2019) is underestimated, with them having a nutritional value that is the same as other planktonic organisms. (Guitierrez *et al*., 2019). To further delve in fungi's role in the Global and Marine Carbon Cycle (MCC), fungi globally influence the flux of biomass-associated carbon. Zoosporic fungi may act as a keystone driver of the oceanic carbon cycle. Also, fungi suspended in the water column process organic matter derived for phytoplankton (Amend *et al*., 2019; Gladfelter *et al*., 2019). Other roles include mineral weathering, arsenic, and manganese cycling, symbiont with chemoautotrophic prokaryotes in deep ocean crust fungi that decompose matter, and a fundamental role in cycling anthropogenic sources of carbon (Amend *et al*., 2019). Intriguingly, fungi may have a future role in coral reefs, where the last resources for thermos-susceptible coral species are

derived from experimental evolution and probiotic microbial transplants (Glatfelter *et al*., 2019). Finally, the ability of fungi to metabolise and degrade recalcitrant polymers (hydrocarbons), while being the first living thing to degrade lignin, lead to the thought: can they be used to potentially degrade plastics, hence be the solution for the Great Pacific Garbage patch or even help decontaminate oil petroleum spills sites (Gladfelter *et al*., 2019)

Lipid biomarkers in fungi is not a novel topic with researches using ergosterol as a biomarker for fungal biomass in atmospheric aerosols (Lau *et al*., 2006), a lipid biomarker of cordycepin accumulation in cordycepin-producing fungi (Qin *et al*., 2019) and abundance of fungi in relation to soil salinity (Barin *et al*., 2013). Lipid biomarkers are a good alternative to other analysis methods as they do not require the isolation and culture of fungi (Summons *et al*., 2022), and it is more useful than DNA as the biomarker can be equated to the biomass (Stahl and Darkin, 1996; Lau *et al*., 2006; Gutarowska and Żakowska 2009; Mansoldo *et al*., 2020). DNA only gives relative abundance in percentage of microbes in environmental samples (Udomsinprasert *et al*., 2021). Assessment of fungi fatty acids and sterols exist (Gladfelter *et al*., 2019) with very recent papers still stating ergosterol is found in all fungi. Direct quotes include: "Ergosterol is a sterol that resides on the cell membranes of fungi" (Marek and Timmons, 2019), "Ergosterol is the major product of sterol biosynthesis in fungi (Bell, 2007), ''Provitamin D_2 (ergosterol), found in plants and fungi…" (Hirsch, 2011). Named as a biomarker (Klein *et al*., 2021), recent studies have shown that ergosterol might not be a suitable biomarker as it is not produced by all fungi (Gutierrez *et al*., 2020). In fact, it has been found absent in chytridiomycetes, a basal lineage of fungi that can be seen in some marine environments (Gutiérrez *et al*. 2016, Hassett *et al*. 2019), and papers on anoxic fungi strolls compositions are missing. The synthesis of ergosterol and unsaturated acids such as 18:2w6, another fungal biomarker (Frostegard and Bååth, 1996) requires oxygen (Summons *et al*., 2006; Ridgway *et al*., 2008). It has also been shown that ergosterol has a linear relationship with biomass (Stahl and Darkin, 1996; Mansoldo *et al*., 2020). This begs the question if that linear relationship is preserved when you deprive the cell of oxygen and whether ergosterol can be synthesized when it is not present.

Gladfelter *et al*. (2019) suggests that the marine fungi identified so far are around 1%. Papers on fungi in anoxic zones are scarce (Dawson and Pace, 2020), with the ones released being focused on fungal diversity (Nagano *et al*., 2010; Raghukumar, 2012), anoxic metabolism of nitrate (Zhou *et al*., 2002), or chemistry, medical and biotechnological potentials (Imhoff, 2016; Pang *et al*., 2016; Bovio, 2019). A suggestion of different classification was proposed in the early 2000s when a facultative anaerobe *Fusarium oxysporum*, preferred aerobic respiration when O_2 was sufficient. First eukaryotic one to do so (Zhou *et al*., 2002). Similarly, when the conditions are anoxic the fungi perform ammonia

fermentation (nitrate mechanism that is metabolic) that supports growth (Dawson and Pace, 2020) This begs the question if ergosterol and FAMEs composition will change in anoxic conditions or of its presence will remain unaffected, indicating another synthesis pathway (Jeennor *et al*., 2006; Summons *et al*., 2006). With anoxic zones being understudied, and ergosterol's label as a biomarker, comes the importance of this research. The aim of this study is to observe a difference in fatty acids and ergosterol composition in the absence of oxygen, and question ergosterol's label as a biomarker. In order to achieve this aim, fungi will be grown separately in oxic and anoxic media, avoiding contamination. Fatty acid methyl esters and sterol profiles will be compared to test the hypothesis that ergosterol and FAMEs change in anoxic environment.

Methods

2.1 Fungi isolation and preparation for biomass analysis

Anoxic Sediment samples were collected using a small syringe. The sediment was suspended in sterile seawater, filtered into different size fractions, and spread on plates using inoculation loops (Cunliffe and Cooper, personal communication). Fungi were left to grow for two to three weeks. *Mucor circinelloides* was isolated inside an anaerobic chamber and identified using DNA barcoding.

Cultures of *Mucor circinelloides* were grown in malt extract media (MEM), prepared using malt extract (15g/L, Sigma Aldrich), sea salt (35 g/L, Sigma Aldrich), agar (15 g/L, Sigma Aldrich), and chloramphenicol antibiotic (0.2g/L in molecular grade ethanol, Sigma Aldrich) post autoclave. 10 anaerobic and 10 aerobic plates were prepared using 30ml of agar mixture and fungal material. 'Soup' solution for liquid cultures was prepared by inoculating 25 ml of liquid MEM (no agar) and fungal material. 45 ml of liquid media and 1 ml of soup were added to 10 pre-autoclaved bottles. Five anoxic conditions and five oxic conditions. Anoxic bottles were saturated under nitrogen gas and sealed with aluminium caps (Figure 1). Incubation of both plates and bottles was conducted at 15 degrees in a 100rpm rotary shaker. Cultures were grown for seven days. Bottle solutions were centrifuged for 6 minutes at 3500 spm thrice, washed with Mili Q and freeze-dried. (Kohlmeyer and Kohlmeyer, 1979).

Figure 1: Pictures of anoxic and oxic liquid culture bottles

2.2 Microscopy

0.5 ml of liquid media bottles was transferred to Eppendorf tubes (10 in total). The solution was diluted with Mili Q by x10. The diluted tubes were put in a vortex and centrifuged, the samples were observed under a magnification factor of 20 on grid slides.

2.3 Extraction of lipid:

The freeze dried samples in foil packets were measured and the dry biomass transferred to pre-ashed glass tubes. A Modified Bligh Dyer Extraction (BDE) method (Rutters *et a*l., 2002; Strut *et al.*, 2004) was used for the extraction of Total Lipids. 10ul of 1mg/ml internal standard was added to each sample prior to extraction. 0.9g of K_2HPO_4 (Sigma Aldrich) and 104 ml of bidistilled water were used for the preparation of a phosphate buffer (P- buffer). Lipids were extracted by sonification for 10 minutes with 4ml of BDE solvent: methanol (100ml), dichloromethane DCM (50ml) and phosphate buffer (40ml) (2:1:0, 8 by volume), followed by centrifugation at 3000spm for 2 minutes, repeated three times. Phase separation was improved by the addition of DCM and P-buffer to achieve ratios of 1:1:0.9, followed by centrifugation at 3000spm for one minute. The lower solvent phase (DCM layer) was pipetted into a pre-weighed glass vial and put under nitrogen stream to evaporate the organic phase, repeated three times. Lipid extract was weighted and stored at -20°C degrees.

2.4 Fatty Acid methyl esters and Sterol

Lipid extracts were split for fatty acids and sterol analysis using 1.8g of potassium hydroxide (KOH) and 32 ml of MeOH. The solution was refluxed in a heating block for an hour at 70°C and left to cool. 2ml of Mili Q water was added to the mixture and extracted three times with DCM. Hydrochloric acid was used to adjust the PH. Solution was then extracted with DCM and dried under nitrogen stream, thrice. Fatty acids were converted to methyl esters with 0.5 ml of Boron Trifluoride in methanol (BF_3 ● CH_3OH) for 10 minutes at 70°C. 0.5 ml Mili Q was added to the mixture and the FAMEs were extracted with 1ml of hexane, repeated 3 times. Sterols were converted to trimethyl silyl esters (TMS) by heating dried extracts with 10ul

BSTFA (N, O-Bis (trimethylsilyl) trifluoroacetamide with 1% TMCS) for 1 hour. Any remaining BSTFA was evaporated under a stream of nitrogen. Samples were re-dissolved in hexane for GC analysis.

2.5 GC MS

An Agilent 7890A GC coupled to an Agilent 5975C Inert XL MSD was used for GC MS. 1 µl of the sample was injected by an automatic injector onto an Agilent DB-5ms (30 m × 0.25 mm × 0.25 µm) column in spitless ion mode. The choice for carrier gas was helium (1 ml min^{-1}; constant flow mode). The programming of The Agilent 7890A GC oven included heating from 70°C (1 min hold) to 130°C at 20°C min^{-1}, followed by a 5°C min^{-1} ramp to 300°C (15 min isothermal hold). The transfer line was held at 250°C, the source at 230°C and the quadrupole at 150°C. Mass spectra were acquired at m/z 50–650.

2.6 GC FID

Agilent 7890A GC fitted with flame ionisation detector (FID) was used for the quantification of fatty acids and sterols. The GC oven was programmed as previously mentioned and Helium was chosen for carrier gas again. Quantitative data was produced by comparing Peak areas detected by GC FID to areas of known concentrations of an internal standard. The internal standard used for this work was 1, 2-diheneicosanoyl-sn-glycero-3-phosphocholine (PC 21:0).

2.7 Data analysis:

GC FID and MS data were both analysed using Openchrom. Data was normalized against an Internal Standard of known concentration in Excel (Figure 2). Fatty acid methyl esters and sterol were calculated in lipid mg/biomass. A Wilcox test in R was performed to determine significant difference between non oxygen and oxygen FAMEs and sterol compositions. Normality and Homogeneity of biomass (mg) was tested using Shapiro and Levene test. Significance between biomass produced in oxic and anoxic environment was tested using one way Anova.

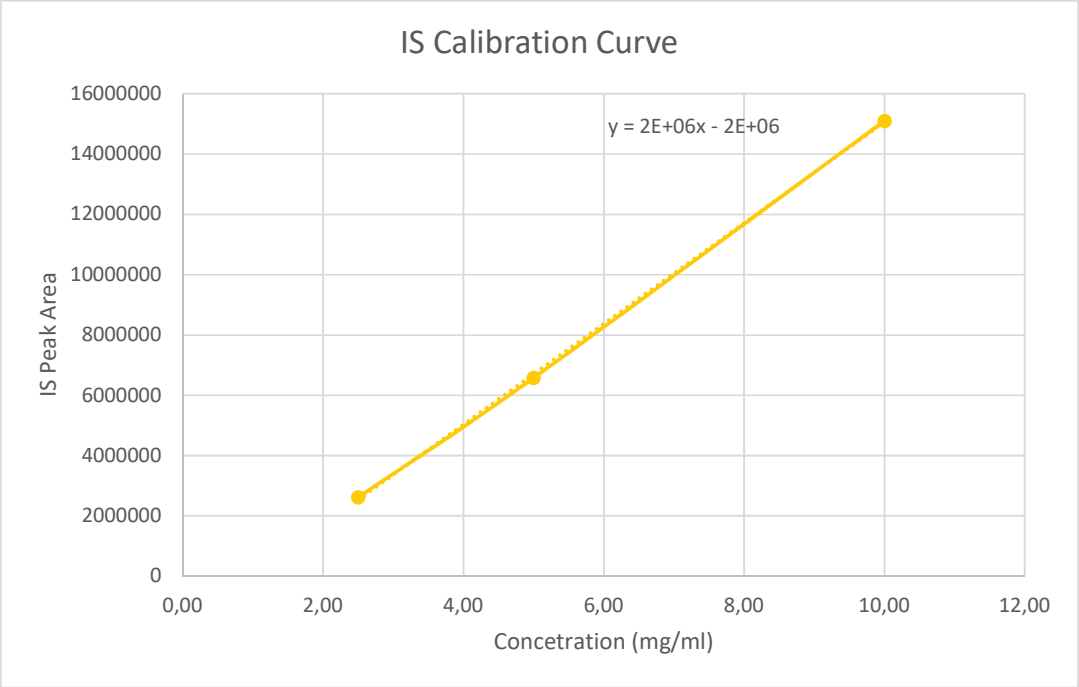

Figure 2: Scatter plot of Internal Standard Calibration Curve showing Internal Standard of known concentrations 2.5, 5 and 10 (mg/ml), against peak area.

Results

Substantial difference in biomass between the oxygen and no-oxygen samples was observed which led to $+O_2$ samples being split in half and only that half biomass recorded to provide more uniform results (Figure 3). Biomass data was normally distributed (W=0.96, p>0, 05) and homogeneity of variance was met ($F_{1,8}$=0.28.0, p=0.61). Significantly more biomass was produced in the oxic environment than the anoxic ($F_{1,8}$=29, p=0.000657)

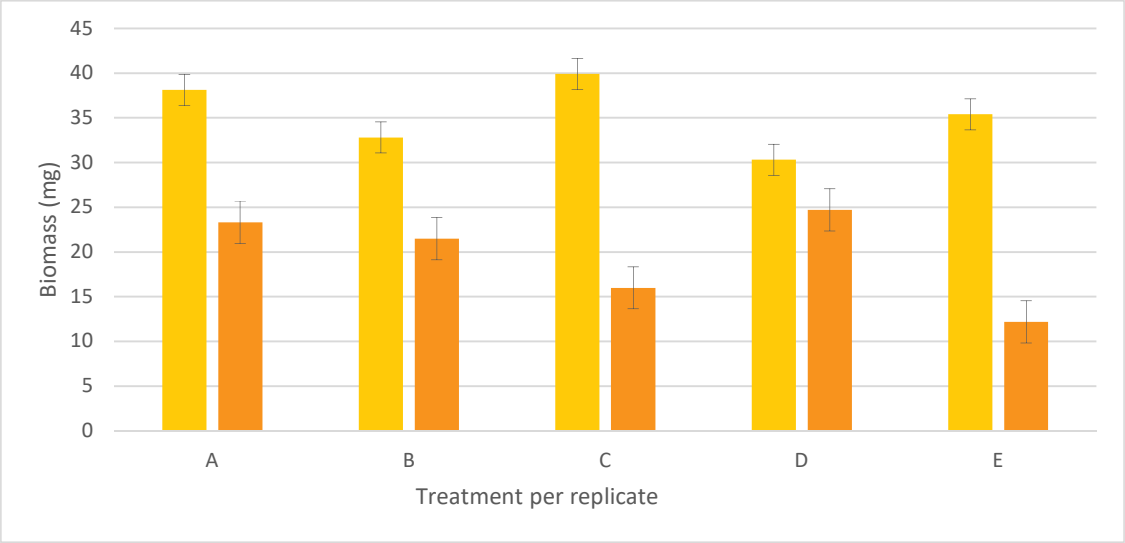

Figure 3: Biomass produced in miligrams in both conditions: oxic (yellow) and anoxic (orange) for each replicate, including standard error

Similarly, there were significantly more FAMEs and sterols in the oxygen treatment (W=2789, $p<0.001$) with mean FAMEs and sterol values being 0.12 and 0.60 lipid mg/biomass of no oxygen and oxygen treatment, respectively. An 11x increase in egrosterol and FAMEs in replicate C oxic was observed and 5x in the other replicates. (Figure 4). Most prevalent acid in the oxygen treatment in all replicates was Octodecenoate (18:1) followed by Octadecatrianoate (18:3) and Hexadecanoate (16:0). Octadecatrianoate and 18:2w6 were much less present in the anoxic environment. In there, highest abundance observed was Hexadecanoate (16:0) followed by Octodecenoate (18:1) and Tetradecanoate (14:0). Unusual result to note was the presence of Pentadecanoate in both conditions.

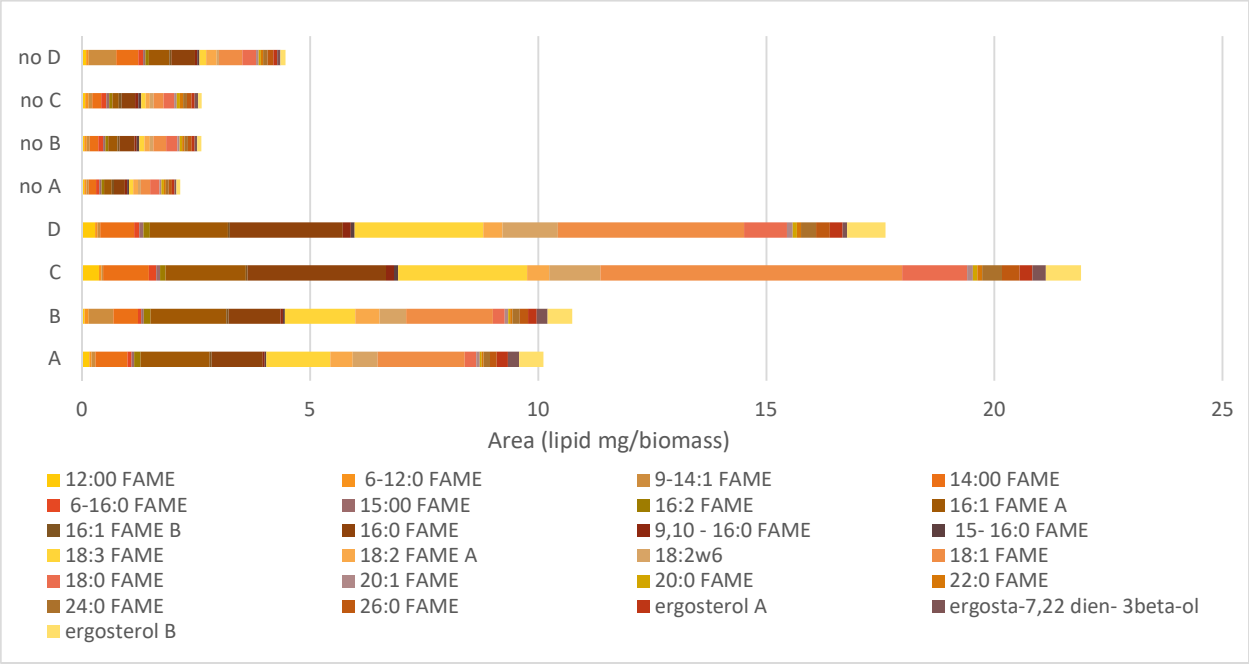

Figure 4: Barplot of fatty acids methyl esters and sterols found in lipids in marine fungi under oxic (replicates: A, B, C, D) and anoxic (replicates: no A, no B, no C, no D) conditions in lipids mg/ biomass

Unsaturated FAMEs accounted for more than the half of lipid composition in the oxygen treatment (61%). Out of these 61%, majority were single double bond, 16% two double bonds and 23% three double bonds. On the other half, the anoxic environment produced less than the half with only 41% of unsaturated fatty acids. Out of them, 69% were single double bonded, 22% two double bonded and 9% three double bonded. Ergosterol accounted for 80% and 73% of sterols in the oxic and anoxic environment respectively (Figure 4). Its isomer ergo-7, 22 diesn-3beta-ol accounting for the rest. Significantly less ergosterol was seen between both conditions ($W=0.789$, $p<0.05$) with Ergosterol B being 7x more in all replicates in oxygen environment (Figure 5)

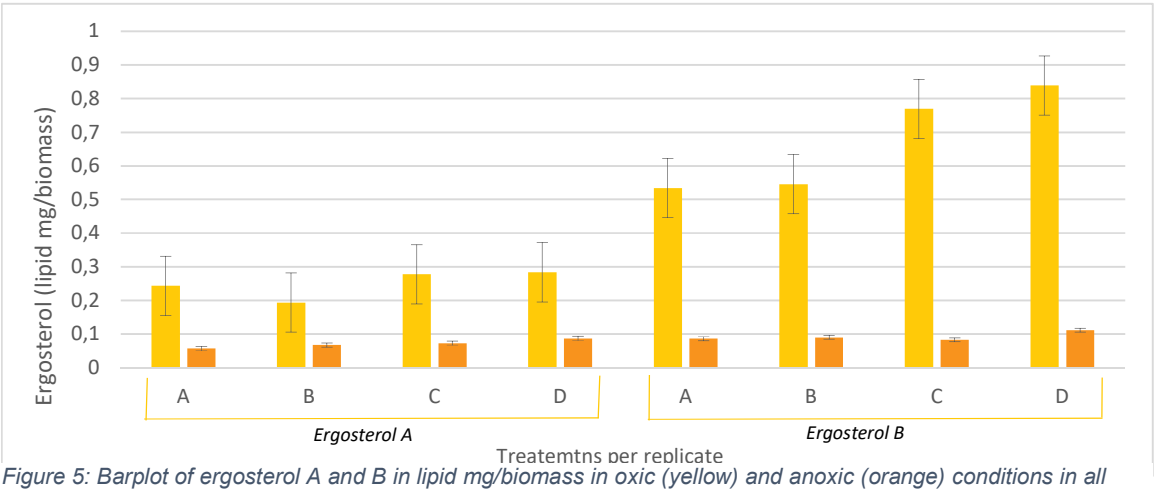

Figure 5: Barplot of ergosterol A and B in lipid mg/biomass in oxic (yellow) and anoxic (orange) conditions in all replicates, including standard error.

Microscopy results showed more budding yeast in the anoxic conditions and more hyphae in in oxic conditions (Figure 6).

a) b)

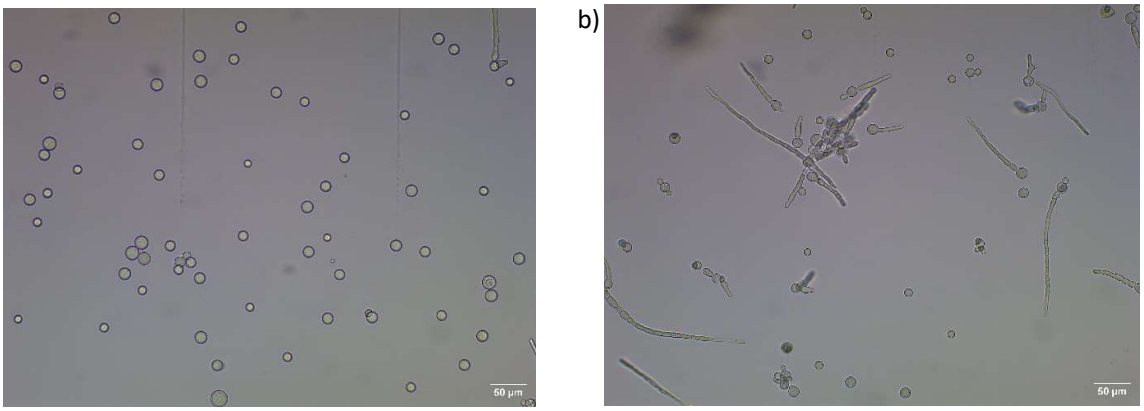

Figure 6: Cultivated Mucor circinelloides in (a) anoxic, (b) oxic environment.

Discussion

The aims were met and the objectives were achieved. The cultures were grown, successfully avoiding contamination and change in fatty acids methyl esters and ergosterol was present. The compared profiles of both treatments supporting the hypothesis right by observing significantly less FAMEs and sterols in the anoxic condition. Further, the findings were supported with visual representation of the treatments and the change it induces via microscopy, where oxygen deprivation induced budding yeast formation (Jeennor *et al*., 2006).We stressed the cell by depriving it of oxygen, preventing the synthesis of unsaturated FAMEs (Ridgway *et al*., 2008), which matches this study's results. When a cell is stressed, an increase in unsaturated fatty acids is seen as response (Montanari *et al*., 2013; Gholinezhad and Darvishzadeh, 2021; Salvatore *et al*., 2023), which was the opposite of what our study showed. Yet, other researches have produced similar results. Upon stressing an algal cell Pushpakumari *et al* (2018) observed decrease in biomass, and in unsaturated fatty acids. Gholinezhad and Darvishzadeh (2013) observed more fatty acids in normal level of stress, but when that level of stress was increased (drought stress) the fungal cell's unsaturated fatty acids rapidly decreased. Therefore, the assumption that depriving the cell of oxygen is an extreme form of stress and the production of less unsaturated FAMEs can be made and that, it is in fact, a stress response. Jeennor *et al*. (2006) performed an almost identical research on *Mucor rouxii* by looking at fatty acids profiles in different stress conditions, one of which was oxic and anoxic environments. Similar results were observed, anoxic conditions that led to decreased long-chain fatty acids, both saturated and unsaturated. Similarly, ergosterol synthesis requires oxygen (Ridgway *et al*., 2008), therefore

its decrease in the anoxic environment was not surprising. Yet, neither the FAMEs, nor the ergosterol were completely absent. A suggestion for this would be a new metabolism pathway for their synthesis without molecular oxygen. Such idea has been suggested in previous studies, with Summons *et al*. (2006) outlining the intricate, specific requirements for such ergosterol pathway to exist. Jeennor *et al*. (2006) suggests an alternative metabolic pathway that does not require oxygen, which was indicated by the presence of odd-chain fatty acid methyl ester, in our case Pentadecanoate. Peng *et al*. (2013) tested the difference in Ammonia oxidizing archea (AOA) community composition in Oxygen Minimal Zones and surface oxygen zones in two places: Arabian Sea and the Eastern Tropical South Pacific. Significant difference between communities was not found but significance between the sites with temperature being the main factor. Founding AOA at anoxic zones led to a possible explanation of archea were not using molecular oxygen for aerobic ammonia oxidation. They theorize that there is a chance of another process that produces ammonia oxidation without oxygen. AmoA gene was found but ammonia oxidation was not detected indicating another metabolism supports their growth where oxygen and ammonium are not detectable. Their study further supports the idea of anaerobic synthesis for ergosterol. An alternative explanation would be a possible adaptation or evolution of the cell. Galea and Brown, 2009 suggest the idea that sterols synthesis was an adaptation to the increase of atmospheric oxygen in pre-historic times. Their arguments delves into the simultaneous sterol and oxygen evolution, their role in the eukaryotic cells as oxygen sensors, regulators and defence against reactive oxygen species and oxygen itself. If sterols are an evolution response to the presence of oxygen, their disappearance when oxygen is removed would be expected. Ergosterol keeps the fungal cell membrane intact (Rodrigues *et al*., 2018), yet fungi are still present in deep anoxic places (Sergeeva and Kopytina, 2014) with some not synthesizing erogsterol at all (Gutierrez *et al*., 2020). This supports the theory of adaption and evolution, otherwise the cell would not be able to survive in an anoxic environment and fungi would not have been found at such conditions. Antifungal drugs are designed to attack ergosterol (Rodrigues *et al*., 2010; Klemptner, 2014) but the results of this study show that with the removal of oxygen, ergosterol rapidly decreases and the linear equation with biomass is lost, which will lead to antifungal drugs losing their function. The production of drugs rely on the idea that ergosterol is present in all fungi, hence the reason it was labelled biomarker. Sterol is missing in some fungi (Gutierrez *et al*., 2020) and this study further supports the idea of ergosterol as unsuitable biomarker.

While the anoxic media bottles were dried with nitrogen and sealed with aluminium caps, the whole process was then performed in a normal setting, allowing oxygen presence in the anoxic samples. Furthermore, the experiment was not immediately started with anaerobic

cultures, rather, they were grown. Therefore the possibility of there being left over oxygen which led to the ergosterol synthesis is highly likely. Therefore, we recommend future studies focusing on erogosterol synthesis in anoxic conditions, to use an anoxic chamber throughout the whole study and use truly anaerobic fungi. Peng *et al*. (2013) found that habitat and temperature had the greatest effect on archea communities, supporting the idea fungi taken from completely anoxic environment and another habitat can have different sterol and FAMEs composition. Following this research it is recommended the studying of fungi taken from deep anoxic zones such as the Black Sea, where they have already been found (Sergeeva and Kopytina, 2014) and no study on their sterol and FAMEs composition has yet been performed. The results prove beneficial to future biomarker researches and present the possibility of methyl 6 palmitate (6-16:0) and Hexadecenoate (16:1 B) as more suitable ones, since their lipid mg/biomass remained unchanged. We urge future researches to go beyond our methods and attempt different approaches, such as Metratrasncriptomix and observing what genes are downregulated/ upregulated. Finally, the results beg to question the knowledge on fungi and FAMEs, especially ergosterol and 18:2w6 as biomarkers for all fungi. Comparison of this study's results and results from future hypoxic and other unique environments studies is needed to deepen our understanding and further confirm the theories laid out in this article. More habitats, its fungi and their sterol and FAMEs compositions must be studied, including ones already known to lack ergosterol, in order to name new biomarkers, that are truly present everywhere.

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