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Seasonal Microbiological Dynamics of Two Salt Lakes in South-East Nigeria

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Abstract

A Saline lake is a body of water that has salinity greater than 3 g/l (0.3%), while a hypersaline lake is a water body that has the moderate 35 g/l (3.5%) salt of oceans. Hypersaline lakes, could be classified as either thalassohaline (created from of evaporation of seawater which results in sodium chloride as the major salt, and a salinity greater than that of seawater by a factor of 5 - 10, also having a neutral or slightly alkaline pH); or athalassohaline (stems from non-seawater sources with high concentrations of ions such as magnesium and calcium and sundry other ions such as potassium, or sodium in smaller amounts). This work aims at studying the seasonal microbiological dynamics of Uburu and Okposi salt lakes, whose microbiological properties have not been studied till date. A plethora of fungal isolates were recovered from both lakes using various fungal media prepared with the lake water. Isolates were identified using standard microbiological procedures and molecular typing. Halotolerance test was used to classify the isolates according to their abilities to withstand various degrees of salt concentrations. Isolates retrieved in their descending order of salt tolerance include: *Aspergillus flavipes* (13 mm at 40%), *Penicillium citrinum* (10 mm at 40%), *Aspergillus ochraceus* (9 mm at 40%), *Aspergillus nomius* (15 mm at 35%), *Microsphaeropsis arundinis* (12 mm at 35%), *Aspergillus sydowi* (28 mm at 30%), *Penicillium janthinellum* (26 mm at 30%), *Mucor* sp. (13 mm at 30%), *Aureobasidium* sp. (12 mm at 30%), *Trichoderma* sp. (9 mm at 30%), *Alternaria* sp. (22 mm at 25%), *Aspergillus* sp. (18 mm at 25%), *Penicillium* sp. (20 mm at 20%), *Cladosporium* sp. (7 mm at 15%). These isolates were classified as borderline extreme halophiles and moderate halophiles, while no slight halophile was isolated.

Keywords

Thalassohaline, Athalassohaline, Halotolerance, Halophiles, Salt Lakes

1. Introduction

A water body connotes the portion of the earth's surface covered with water (such as a lake, river or ocean). Two kinds of salt water are classified on the earth's surface, and they include epicontinental (inland surface) salt lakes and marine waters (the ocean) [1]. However, this research is slanted towards the latter type, specifically, ephemeral or permanent water bodies with salinities greater than 3 g/L. Lakes are water bodies surrounded by land but are not as a result of distributary from an ocean [2]; they are relatively still water bodies when compared to rivers which are known for flowing waters [3]. They can also contain either salt water or fresh water, and are basically larger than ponds. Water bodies are subject to seasonal variations which impact microbial distributions found in them per time [4]-[6].

Hypersaline lakes, typified by low oxygen concentrations, could either be thalassohaline or athalassohaline (from the Greek word, *thalassa*, meaning sea) [7]-[12]. Thalassohaline lakes are creations of evaporation of seawater and as such contain sodium chloride as the major salt, with a salinity that surpasses that of seawater by a factor of 5 - 10 and a neutral or slightly alkaline pH [13]. Examples include the Great Salt Lake of Utah, playas, salt mine drainage waters, brine springs from underground salt deposits, natural coastal splash zones and tide pools, and solar salterns [14]. Athalassohaline lakes on the other hand stem from non-seawater sources and are made up of high concentrations of ions such as magnesium and calcium and sundry other ions *viz* potassium, or sodium in trace amounts and are often the sources of potash, magnesium metal, soda, and even borax if the waters have high boron content. Good examples are the alkaline soda lakes of Egypt (Wadi El-Natrun), soda lakes of Antarctica, Dead Sea, and Big Soda Lake and Mono Lake in California [14]-[18]. De-salination of salt water bodies using either membrane or thermal technologies have been proposed to be a promising approach to unending water supply in the world [19], and goes to portray the potential usefulness of salt lakes amidst their perceived toxicity.

Uburu and *Okposi* salt lakes are situated in Ohaozara Local Governmeant Area of Ebonyi State, Nigeria and remain under-studied till today. Much of the research done on these lakes by [20]-[23], has only focused broadly on its geology, geochemistry and geophysics with special attention on its physicochemical parameters, hydrochemistry, water types and facie evolution, gross alpha and beta concentrations, ground water exploration using aeromagnetic and electromagnetic geophysical methods, respectively. Unlike other salt lakes across the world, *Uburu* and *Okposi* salt lakes have not been classified as either thalassohaline or athalassohaline. More recent biological studies have only concentrated on the initiation of oxidative stress in the antimicrobial susceptibility of Ebonyi salt mining sites, reproductive parameters and reduction of sperm number, motility and morphology of adult male Sprague-Dawley rats and radiological health risks due to gamma dose rates around these lakes [24]-[28]. However, some scientists have studied the halophilic archaea, bacteria, cyanobacteria and fungi of other hypersaline ecosystems in various countries of the world such as India (Goa solar salterns, Gujarat coast, Kerala and Sambhar salt lakes); Israel/Jordan (Dead sea); USA (Great salt

lake of Utah). None has studied those of *Uburu* and *Okposi* salt lakes of Nigeria.

In 1978, Donn Kushner put forward what remains today as the most acceptable definition and classification of microorganisms based on their salt requirement and tolerance. He divided halophiles into: extreme halophiles (optimum growth at 2.5 - 5.2 M NaCl), borderline extreme halophiles (having their optimum at 1.5 - 4.0 M NaCl), moderate halophiles (preferring media with 0.5 - 2.5 M NaCl), slight halophiles (having optimum growth at 0.2 - 0.85 M NaCl) and halotolerant microorganisms that do not require salt for growth but tolerate salt often in high concentrations; and extremely halotolerant if the salt requirement exceeds 2.5 M NaCl [29]. Again, the mercantile importance of halophiles has been noted and benefitted from since antiquity. For instance, Roman soldiers were given "salt money" (*salarium argentum*) as a part of their payment; and from this practice, the English word "salary" (*sal* meaning salt, *ary* pertaining to or connected with money) was coined. According to evidences from small subunit rRNA sequence-based phylogenetic tree of life, halophiles can be found in each of the three domains of life: Archaea, Bacteria and Eukarya [30]. However, bacterial diversity is greatly decreased with increased salinity, while fungal diversity appears to be more complex, which led to this study being mainly fungal-based. Water chemistry of salt lakes impact on the diversity of microorganisms found in them, with seasons also contributing to the microbial niches per fluctuation in water volumes in both wet and dry seasons. For fungi, they basically have a modified plasma membrane fluidity that enables them alleviate salinity stress and also perform symbiosis with plant rhizospheres or as endophytes [30]. Some of these fungi possess the ability to solubilize insoluble phosphates from calcium, aluminium and iron which plays vital roles in nutrient sampling and remediation of heavy metals. Thus, it becomes important to study the fungal diversity in this extreme environment for reasons centred on possible biotechnology usefulness and climate change modulation at large.

The aim of this study is to investigate the seasonal microbiological changes of halophiles isolated from Uburu and Okposi salt lakes, with a view of specifically investigating and ascertaining the fungal dynamics/variations in both lakes over a one-year period; also to characterize and identify the halophilic molds associated with the lakes, carry out halotolerance evaluation of the isolates and classifying these halophiles accordingly that are believed to have possible cutting edge biotechnological applications.

2. Materials and Methods

2.1. Sample Collection

Water samples for microbial analysis were collected aseptically in sterile screw-capped bottles and delivered to the Laboratory of Applied Microbiology at Nnamdi Azikiwe University in Awka for processing. Twelve (12) water samples were obtained from each lake (Uburu and Okposi), for a total of twenty-four (24) samples. To represent spatial variability, four different sampling locations were randomly chosen at each lake: one from the lake's central deepest point, one from a shallow area near the shoreline with visible sediment disturbance, one from an area with

noticeable human activity (near salt harvesting points), and one from a relatively undisturbed area away from direct human impact. To compensate for micro-scale variation, a total of three water samples were obtained at depths ranging from 15 to 20 cm beneath the surface of the water at each of these four points of interest. Every single sample was collected under an array of precise environmental conditions: water temperature (measured with a digital thermometer dipped into the water), pH (measured with a portable pH meter), salinity (measured with a refractometer), water clarity (visually assessed as clear, slightly turbid, or turbid), and any observable weather conditions (sunny or raining). The exact time and date of sampling for each sample were also precisely recorded.

2.2. Seasonal Microbiological Analyses

Some Microbiological analyses were performed on the salt lake samples on a seasonal basis (twice for both rainy and dry seasons) for a one-year period. These analyses were carried out quarterly from January to December over a one-year period.

2.3. Isolation, Characterization and Identification of Halophilic Fungal Species

2.3.1. Fungal Isolation and Characterization

The water samples obtained from the lakes quarterly from January to December were stored in ice chests and transported to the laboratory before being transferred to refrigerators. Exactly 0.1 ml of the water samples were transferred into the centre of already prepared agar plates using sterile pipettes. With the aid of a sterile glass spreader, the aliquot was spread evenly on the surface of the agar plate. All fungal media were amended with 0.5 mg/ml of Chloramphenicol to inhibit bacterial growth. Plates were incubated at room temperature for 10 days each. Developing fungal isolates were purified by repeated subculture technique and transferred to Bijou bottles with agar slopes for identification and storage. Czapex-Dox Agar (CzA) and SDA prepared with the lake water were used for the isolation of halophilic fungal species.

2.3.2. Identification of Fungal Isolates

Preliminary fungal characterisation was carried out using the slide culture and wet mount techniques to examine their cultural and microscopic features with reference to the Manual of Fungal Atlases according [31]-[34]. Thereafter, ITS-region sequencing was used to confirm identities of the 7 most halophilic isolates at Macrogen Inc., 10 F, 254 Beotkkot-ro, Geumcheon-gu, Seoul, Republic of Korea.

A proprietary fungal culture formulation [microLYSIS®-PLUS (MLP), Microzone, UK] was subjected to the rapid heating and cooling of a thermal cycler, to lyse cells and release deoxyribonucleic acid (DNA). Following DNA extraction, Polymerase Chain Reaction (PCR) was used to amplify copies of the rDNA *in vitro*. The PCR product quality was assessed using gel electrophoresis. PCR purification step was performed to remove unutilized dNTPs, primers, polymerase and other PCR mixture compounds, and obtain a highly purified DNA template for sequenc-

ing. Sequencing reactions were undertaken using BigDye® Terminator v3.1 kit from Applied Biosystems (Life Technologies, UK) which utilises fluorescent labelling of the chain terminator ddNTPs, to permit sequencing. Removal of excess unincorporated dye terminators was done to ensure a successful electrophoresis of fluorescent-labelled sequencing reaction products on the capillary array AB 3130 Genetic Analyzer (DS1) DyeEx™ 2.0 (Qiagen, UK). Modules containing pre-hydrated gel-filtration resin were optimized for clean-up of sequencing reactions containing Big Dye® terminators. Dye removal was followed by suspension of the purified products in highly deionized formamide Hi-Di™ (Life Technologies, UK) to prevent rapid sample evaporation and secondary structure formation. Sample was loaded onto the AB 3130 Genetic Analyzer and sequencing undertaken to determine the order of the nucleotide bases in the DNA oligonucleotide. After sequencing, identifications were done by comparing the obtained sequences with those available from the European Molecular Biology Laboratory (EMBL) database. The strains were identified using Inter specific region sequencing analyses [35].

2.3.3. Halotolerance Test of the Isolates

Developing fungal cultures were inoculated on CzA amended with concentrations of 0, 5, 10, 15, 20, 25, 30, 35, 40, 45, and 50 % w/v NaCl. Growth was observed and noted after 7 days of incubation in terms of colony diameter (mm). Plates that failed to show growth up to 7 days of incubation were further incubated till the fifteenth day to factor in possible delayed growth of isolates. Thereafter, salt tolerance was determined by plotting the colony diameter of the molds in millimetres against percentage salt concentration in the culture medium [36]. Isolates were classified as slight halophiles (0.2 - 0.5 or even 0.85 M salt equivalent to 1% - 5%), moderate halophiles (0.5 - 2.5 M or 0.85 - 3.4 M salt equivalent to 5% - 20%), borderline extreme halophiles (1.5 - 4.0 M salt equivalent to 9% - 23%) and extreme halophile (2.5 - 5.2 M salt equivalent to 15% - 30%) according to various classification schemes proposed by [37] [38].

3. Results

1) Study Locations

The images of Uburu and Okposi Salt lakes are presented in **Plate 1** and **Plate 2** as shown below.

2) Seasonal Microbiological Analyses/Dynamics of Uburu and Okposi Salt Lakes

Fourteen fungal isolates labeled UBA, UBD, UBE, OKA, OKC, UA, UB, UC, OA, OD, UE, OKB, OB, and OKPb were obtained from the lakes and characterized. All isolates were identified colonially and microscopically. Thus, seven best salt tolerant isolates were selected were identified to species level using ITS rDNA sequencing tool as shown in **Table 1** and **Table 2**. The best salt tolerant fungus was selected for further studies. However, it can be concluded from **Table 1** and **Table 2** that fungi belonging to the genera of *Aspergillus* and *Penicillium* occurred in both lakes throughout the study period.

Plate 1. Uburu salt lake.

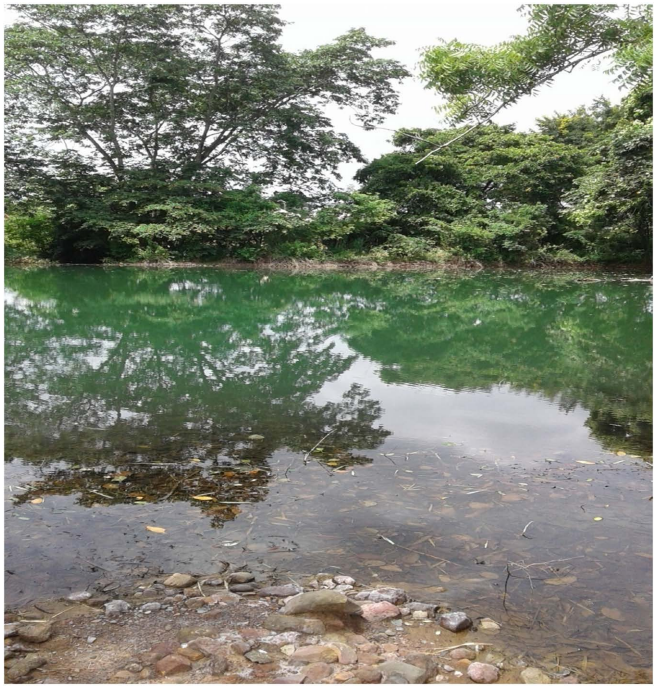

Plate 2. Okposi salt lake.

3) Halotolerance Test of the Isolates

Recovered isolates are arranged in descending order of salt tolerance *viz*: *Aspergillus flavipes* (13 mm at 40%), *Penicillium citrinum* (10 mm at 40%), *Aspergillus ochraceus* (9 mm at 40%), *Aspergillus nomius* (15 mm at 35%), *Microsphaeropsis arundinis* (12 mm at 35%), *Aspergillus sydowi* (28 mm at 30%), *Penicillium janthinellum* (26 mm at 30%), *Mucor* sp. (13 mm at 30%), *Aureobasidium* sp. (12 mm at 30%), *Trichoderma* sp. (9 mm at 30%), *Alternaria* sp. (22 mm at 25%), *Aspergillus* sp. (18 mm at 25%), *Penicillium* sp. (20 mm at 20%), *Cladosporium* sp. (7 mm at 15%) as seen in Figures 1-14. From the above conclusions, it can be inferred that *Aspergillus flavipes*, *Penicillium citrinum*, *Aspergillus ochraceus*, *Aspergillus nomius*, *Microsphaeropsis arundinis*, *Aspergillus sydowi*, *Penicillium janthinellum*, *Mucor* sp., *Aureobasidium* sp., *Trichoderma* sp., *Alternaria* sp., and *Aspergillus* sp. belonged to the extreme halophiles class, whereas, *Penicillium* sp. and *Cladosporium* sp. belonged to the borderline extreme halophiles and moderate halophiles respectively. No slight halophile was isolated in this work as seen in Table 3.

Table 1. Phenotypic and genotypic characteristics of fungal isolates from Uburu and Okposi salt lakes.

Code	Colony morphology	Microscopy	Identity
UBA	The colonies on SDA had cushion-shaped structures scattered throughout and were dark green with a golden hue. Cultures were characterized by the formation of needle-shaped crystals. The opposite side was tan.	Hyaline, erect, branching conidiophores carried spore masses apically at verticillate phialides, which were thick and short. Conidia were one-celled, globose, subglobose, hyaline, phialosporous, or ovate. The chlamydospores were subglobose and brown.	*Trichoderma* sp.
UBD	Colonies on SDA had a pale greyish-brown texture and were floccose, or cottony. Colonies filled the entire petri dish in three days due to their high growth rate. The opposing side was yellow in color. For five days, colonies were incubated at 30˚C.	The sporangiophores were erect, hyaline, non-septate, and branched circinately and sympodially. Sporangia were dark-brown, terminal, smooth, round, and coarsely echinulate (20 - 80 µm in diameter). Sporangiospores were either pale-brown or hyaline. The ellipsoidal collumellae measured 4.5 - 7 × 3.5 - 5 µm. And there were no chlamydospores.	*Mucor* sp.
UBE	Colonies on SDA grew at a moderately slow rate at 30˚C. texture ranges from floccose (woolly tufts of soft "hairs") to velutinous (soft, velvety surface). Radially sulcate colonial growth was characterized by deep, narrow furrows or radial grooves that resembled a wheel's spokes. The mature colony had a white periphery (outside edge) and a core color that ranged from greyish-turquoise to greyish-orange. Exudates (extrolites) were commonly generated and showed up as liquid droplets on the colony's surface. They could be transparent, pale yellow, or reddish-brown in color. Pale yellow to light golden-brown was the color of the backside.	Septate, hyaline (clear, not pigmented) hyphae were formed by the organism. The stipes of smooth-walled conidiophores were biverticillate and rather lengthy (100 - 300 µm). Metulae were observed in whorls of three to five different structures and ranged in length from 12 to 15 µm. Phialides ranged in length from 7 to 12 µm and had an ampuliform (flask-shaped) shape. Conidia had a smooth or finely roughened surface and ranged in shape from globose to sub-globose (round to off-round) with a diameter of 2.2 to 3.0 µm. Conidia create rather lengthy chains and are resistant to disturbance. These two traits set Penicillium citrinum apart: the conidia were spherical and formed in distinct chains, and the metulae were longer than the phialides.	*Penicillium citrinum*
OKA	The organisms on SDA were light yellow to yellow to dull white. The other side had a dark brilliant yellow color. The growth rate was satisfactory and no sclerotia were seen.	The conidiophore measured 403 - 521 µm in length and 7.3 - 8.7 µm in width. Conidiophores were coarsely roughened and ranged in color from yellowish to pale brown. Conidiospores were ellipsoid, smooth to finely roughened, and ranged in size from 2.5 to 2.7 µm. The diameter of the vesicle is 20 - 24 µm. Phialides are biseriate, measuring 2.2 - 2.4 µm in diameter and 7 - 8 µm in length.	*Aspergillus ochraceus*

Continued

OKC	Colonies on SDA were green with white borders. It was cream to light cream-brown on the back. A good growth rate was noted. Coonial has a floccose to silky texture.	Conidiophores are colorless and echinulate. The globose vesicle has globose echinulate conidia and is covered in biseriate phialides. Conidia had fine, rough walls and were subglobose or ellipsoidal, whereas sclerotia were black and bullet-shaped.	*Aspergillus nomius*
UA	Colonies on SDA grew slowly and had extensive aerial mycelium that was first greenish-grey before turning dark brown or grey-brown.	The irregularly formed, septate, pigmented hyphae had enlarged segments up to 4 µm in diameter. The subspherical, 250 - 350 µm diameter pycnidia had a pseudoparenchymatous wall made up of closely spaced cells that gave the appearance of being angular in cross section (textura angularis). Conidiogenous cells are ampulliform and can reach a length of 5 µm. Conidia are cylindrical, brown, 3.5 - 4.5 × 1.0 - 1.5 µm, with thick, smooth walls.	*Microsphaeropsis arundinis*
UB	On SDA agar, colonies have a velvety, light grayish green surface. The opposite side has a light yellowish brown color. Growth rate: 2 - 4 cm in diameter in 10 days following room temperature incubation	Conidiophores are erect, hyaline, and branch penicillately at the apexes. They have terminal phialides, verticillate metula, and catenulate conidia on each phialide, forming somewhat divergent conidial heads. The phialides have abruptly tapered terminals and are pen-pointed. Phialosporous conidia are black in mass, ellipsoidal or subglobose, one-celled, smooth, and apiculate at one end. They are also pale green.	*Penicillium janthinellum*
UC	Growth was reasonably quick on SDA, and after 7 days of incubation, the cell reached maturity. After 7 days of incubation at 25˚C, the colony diameter measured between 1 and 3 cm. The colonies had the following characteristics: they were smooth, flat, resupinate, moist, yeast-like, mucoid to pasty, glossy, and leathery. At first, the surface was white, pale pink, or yellow; as it aged, it turned brown to black, velvety, and had a grayish fringe. The reverse was either black or whitish.	Blastoconidia had a light hue. It was noted that blastoconidia in tufts developed synchronously. Initially hyaline, the septate hyphae eventually turned dark brown as they aged. The hyphae were 2 - 10 µm wide, but they might have been as thick as 15 - 20 µm. Conidiogenous cells were found terminally in the hyphae or intercalary, and they were not highly differentiated. The conidia were hyaline, oval to cylindrical, and one-celled, measuring 4 - 6 × 2 - 3 µm. They were found along the hyphae or in groups. Additionally, chlamydoconidia, arthroconidia, and blastoconidia may be seen. Old, mature cultures developed phaeoid arthroconidia with thick walls and one to two cells.	*Aureobasidium* sp.
OA	Green mycelium on SDA that turns yellowish, drab, buff, and then brownish as it ages. Colonies grew slowly. Orange-brown to red was the reverse.	Conidia chains that were extremely densely packed were seen. The largest vesicles were up to 30 by 40 µm and were subglobose to elliptical; in smaller species, their diameter was often double that of the conidiophore. Primary sterigmata were roughly 6 or 8 µm by 2 to 3 µm, whereas secondary sterigmata were 5 to 8 µm by 1.5 to 2 µm. Sterigmata were in two series, colorless or almost so, and were densely packed over the vesicle's tip in small heads. In ancient cultures, conidia were smooth, subglobose, colorless or almost so at high magnification, and had chains that aggregated to create columns that could be seen with a hand lens. They were 2 to 3 µm in size.	*Aspergillus flavipes*
OD	The growth rate on SDA was moderate. The hue ranged from dark green to blue-green to greyish-turquoise. The reverse was reddish-brown to maroon. The texture was both velutinous (dense, silky hairy) and lanose (woolly). Approximately 200 µm of conidiophore stipes were formed by colonies, giving them a woolly or hairy look.	The conidiophores were carried on long, smooth-walled stipes that were either slightly brownish or hyaline (translucent/colorless). The sub-spherical, pyriform (pear or teardrop shaped) to moderately clavate (club shaped) vesicles ranged in width from 7.0 µm to 17 µm. Metulae (2 µm - 3.5 µm by 4 µm - 6 µm) and phialides (2 µm - 3 µm by 5 µm - 7 µm) were biserate conidiogenous structures. Numerous isolates formed diminutive conidial structures that might mimic the heads of penicillates (like Penicillium). The round, echinulate, or spinose (rough, jagged texture) conidia ranged in diameter from 2.5 µm to 4.0 µm. There were also Hülle cells.	*Aspergillus sydowi*

Continued

UE	The cultures on SDA were funiculose with bundles of hyphae, fluffy, brilliant yellowish green with a bluish green tint, and reverse yellowish pink with a reddish purple tint. Growth is fairly good.	Conidiophores were erect, hyaline, and branched penicillately at the apexes with primary and secondary metula. They were formed from aerial hyphae. The phialides were lanceolate or suddenly sharpened, and each phialide had open-spaced bright green conidial heads with verticillate phialides and catenulate conidia. Conidia were black in mass, globose to subglobose, phialosporous, one-celled, and minutely echinulate on the surface. They were also pale green.	*Penicillium* sp.
OKB	Colonies on SDA are suede-like to floccose, black to olivaceous-black or greyish, and grow quickly. Due to the formation of pigment, the backside was brown-black.	Under a microscope, simple, occasionally branched, short or elongate conidiophores were sympodially transformed into branched acropetal chains (blastocatenate) of multicellular conidia (dictyoconidia). Conidia are pale brown, smooth-walled, verrucose, obclavate, obpyriform, ovoid to ellipsoidal, and may have a short conical or cylindrical beak. Because Alternaria produces the pigment melanin, its structures have dark septate hyphae and appear brown to black in color.	*Alternaria* sp.
OB	Colonies on SDA were initially white, flat, and powdery before aging to a yellowish-brown color. The opposing side was yellow in color. For five days, colonies were incubated at 30˚C.	Conidiophores had a blue appearance and ended in a uniseriate phialide vesicle. Conidia had a single cell, rough walls, and were formed in lengthy, diverging chains.	*Aspergillus* sp.
OKPb	At room temperature, the growth rate on SDA was mediocrely decent, and the texture ranged from silky to powdery. It was black on the back and olivaceous green to black on the front.	Conidia had black hila, were elliptical to cylindrical, and ranged in color from pale to dark brown. They were found in easily disarticulating branching chains. The conidial wall was either smooth or echinulate at times. Their conidia were unicellular.	*Cladosporium* sp.

Table 2. Seasonal dynamics of micro-organisms isolated from Uburu and Okposi salt lakes.

Quarters	Months	Uburu	Okposi
First	January-March	*Penicillium* sp. *Alternaria* sp. *Penicillium janthinellum* *Aspergillus flavipes*	*Aspergillus flavipes* *Aspergillus sydowi*
Second	April-June	*Microsphaeropsis arundinis* *Penicillium janthinellum* *Aureobasidium* sp. *Penicillium citrinum* *Aspergillus flavipes* *Trichodema* sp.	*Aspergillus sydowi* *Aspergillus flavipes* *Aspergillus* sp.
Third	July-September	*Trichoderma* sp. *Mucor* sp. *Penicillium citrinum* *Aspergillus* sp.	*Aspergillus ochraceus* *Aspergillus nomius*
Fourth	October-December	*Aspergillus* sp. *Cladosporium* sp.	*Aspergillus ochraceus* *Penicillium citrinum*

Table 3. Halophilic classification of isolates.

Slight Halophiles (1% - 5% Salt)	Moderate Halophiles (5% - 20% Salt)	Borderline Extreme Halophiles (9% - 23% Salt)	Extreme Halophiles (15% - 30% Salt)
	Cladosporium sp. (7 mm at 15%)	*Penicillium* sp. (20 mm at 20%),	*Aspergillus flavipes* (13 mm at 40%)
			Penicillium citrinum (10 mm at 40%)
			Aspergillus ochraceus (9 mm at 40%)
			Aspergillus nomius (15 mm at 35%)
			Microsphaeropsis arundinis (12 mm at 35%)
			Aspergillus sydowi (28 mm at 30%)
			Penicillium janthinellum (26 mm at 30%)
			Mucor sp. (13 mm at 30%)
			Aureobasidium sp. (12 mm at 30%)
			Trichoderma sp. (9 mm at 30%)
			Alternaria sp. (22 mm at 25%)
			Aspergillus sp. (18 mm at 25%)

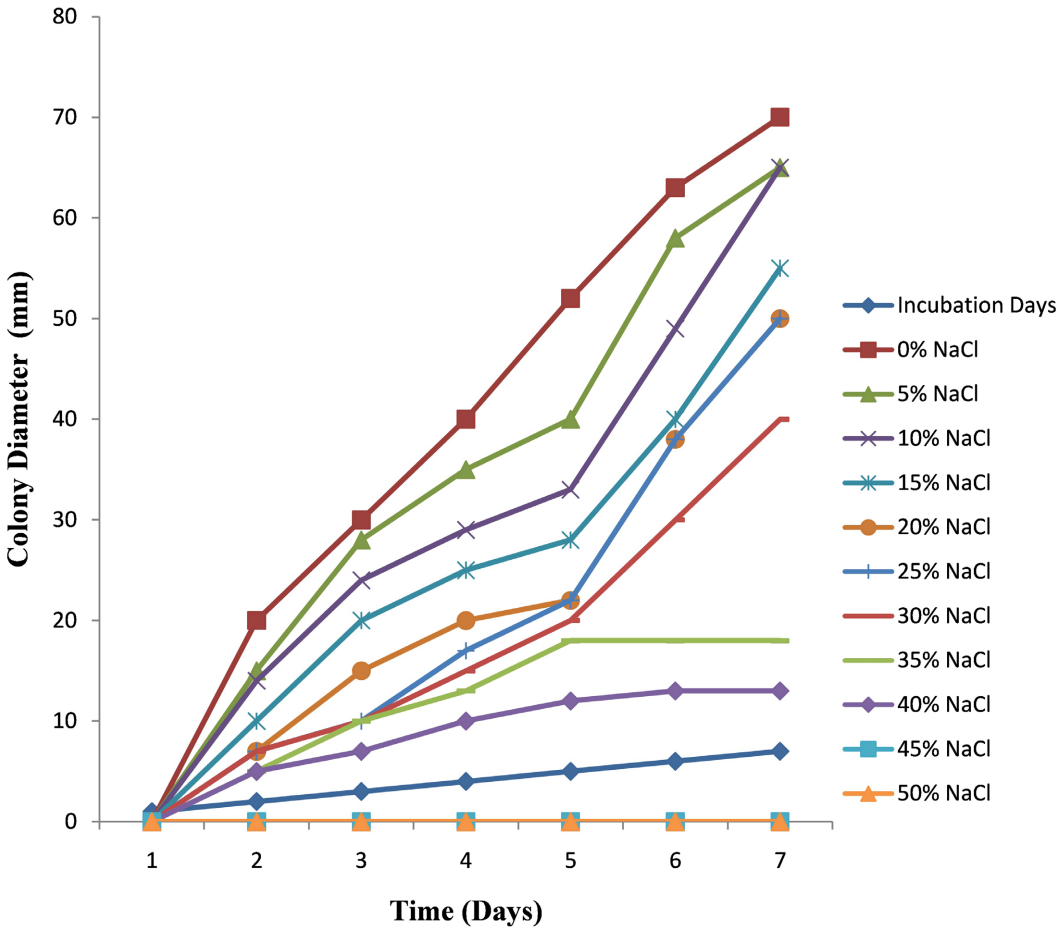

Figure 1. Halotolerance curve of *Aspergillus flavipes*.

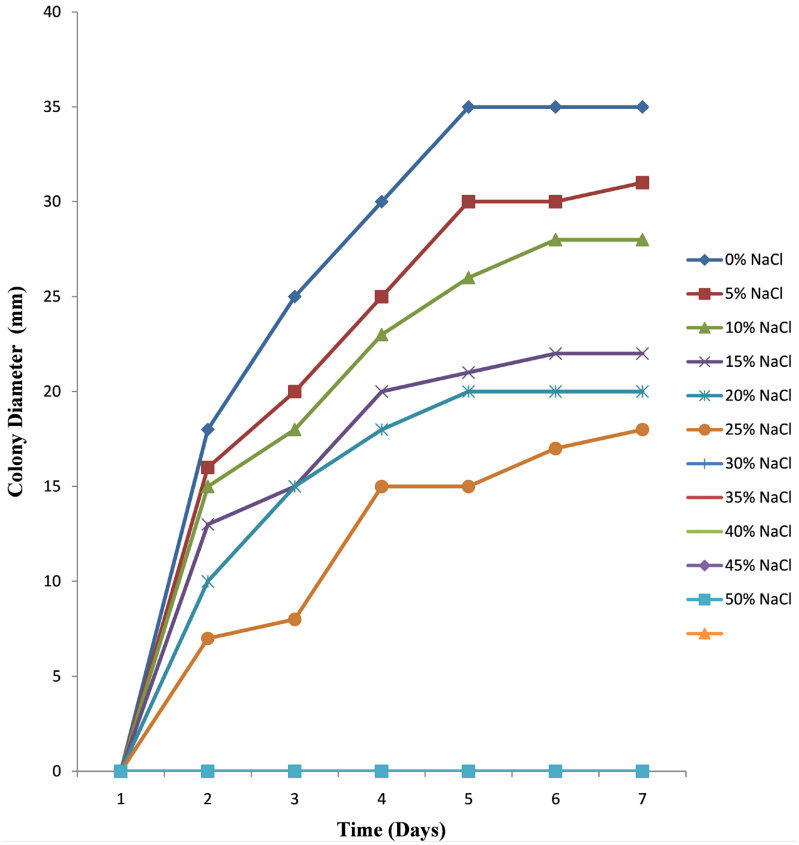

Figure 2. Halotolerance curve of *Aspergillus* sp.

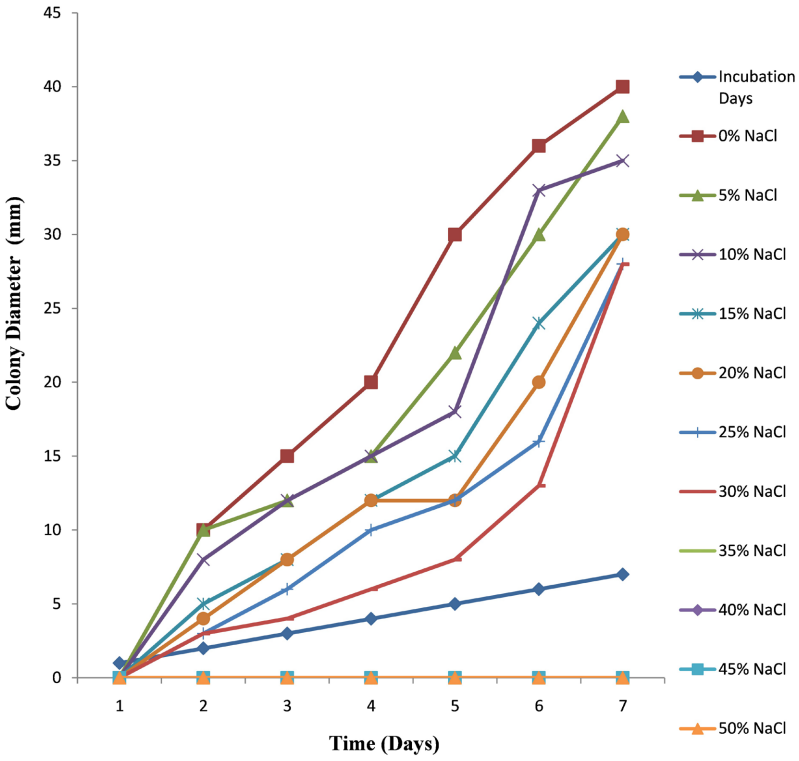

Figure 3. Halotolerance curve of *Aspergillus sydowi*.

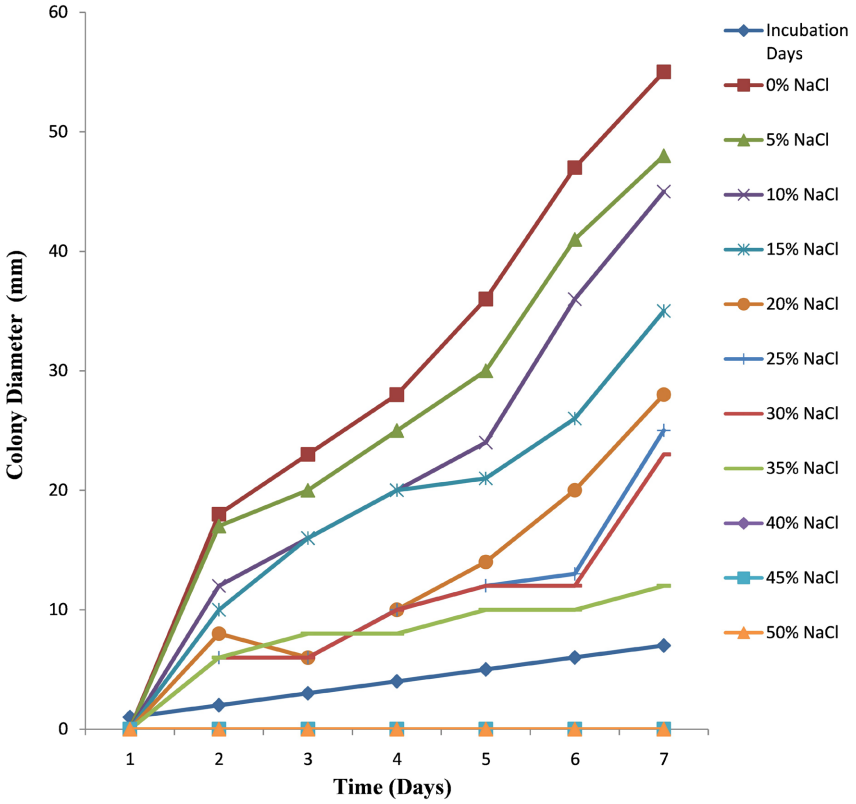

Figure 4. Halotolerance curve of *Microsphaeropsis arundinis*.

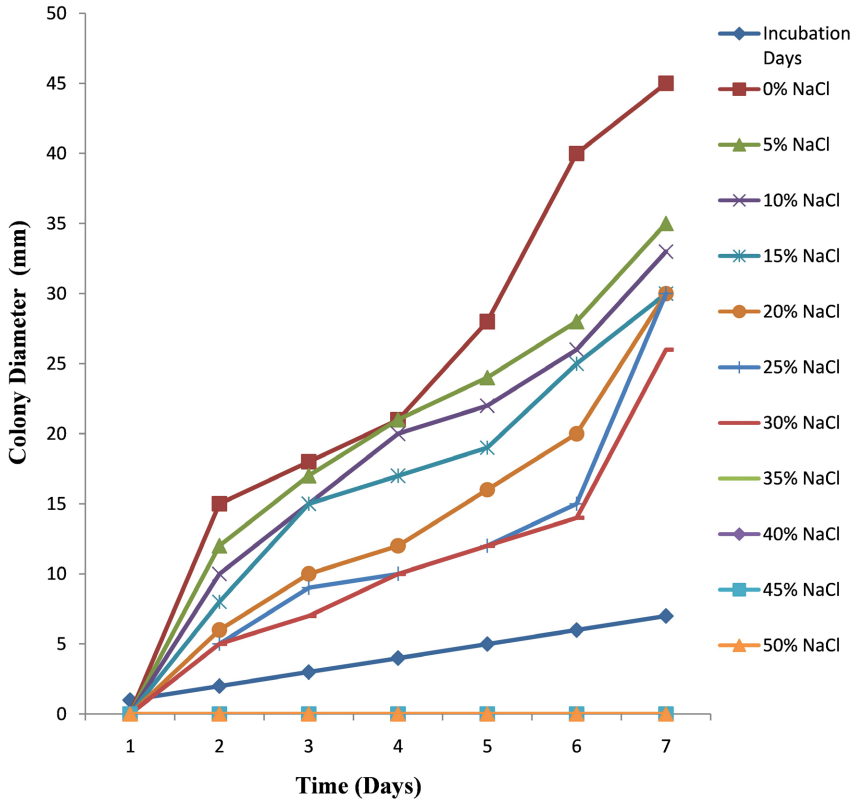

Figure 5. Halotolerance curve of *Penicillium janthinellum*.

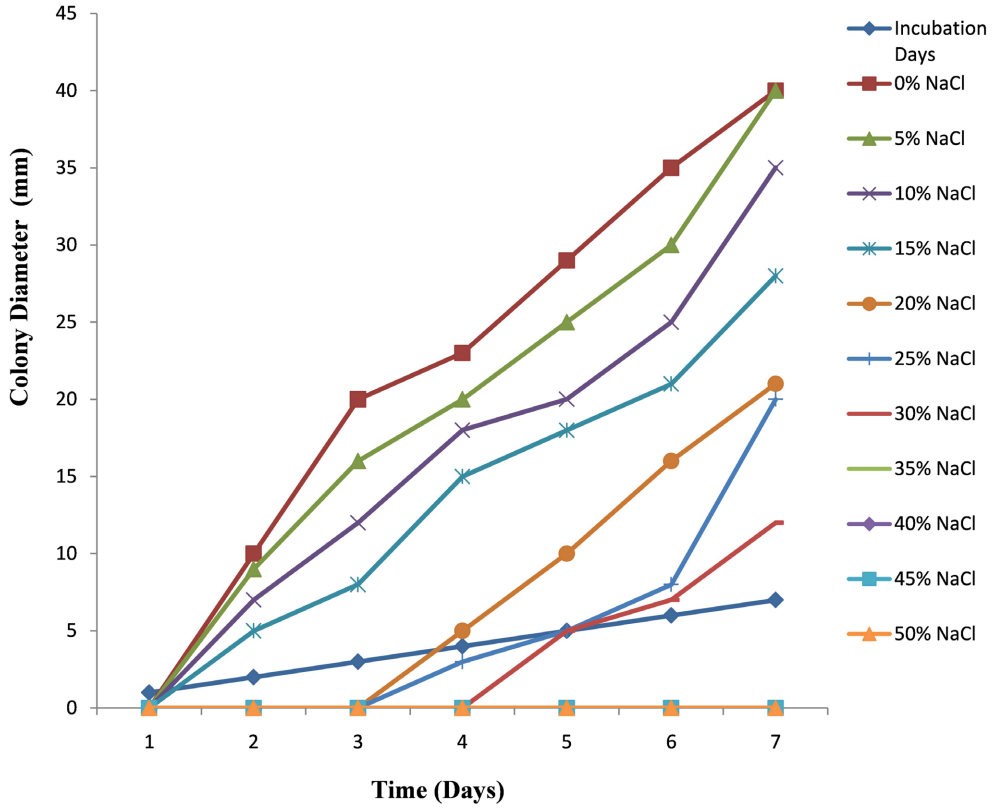

Figure 6. Halotolerance curve of *Aureobasidium* sp.

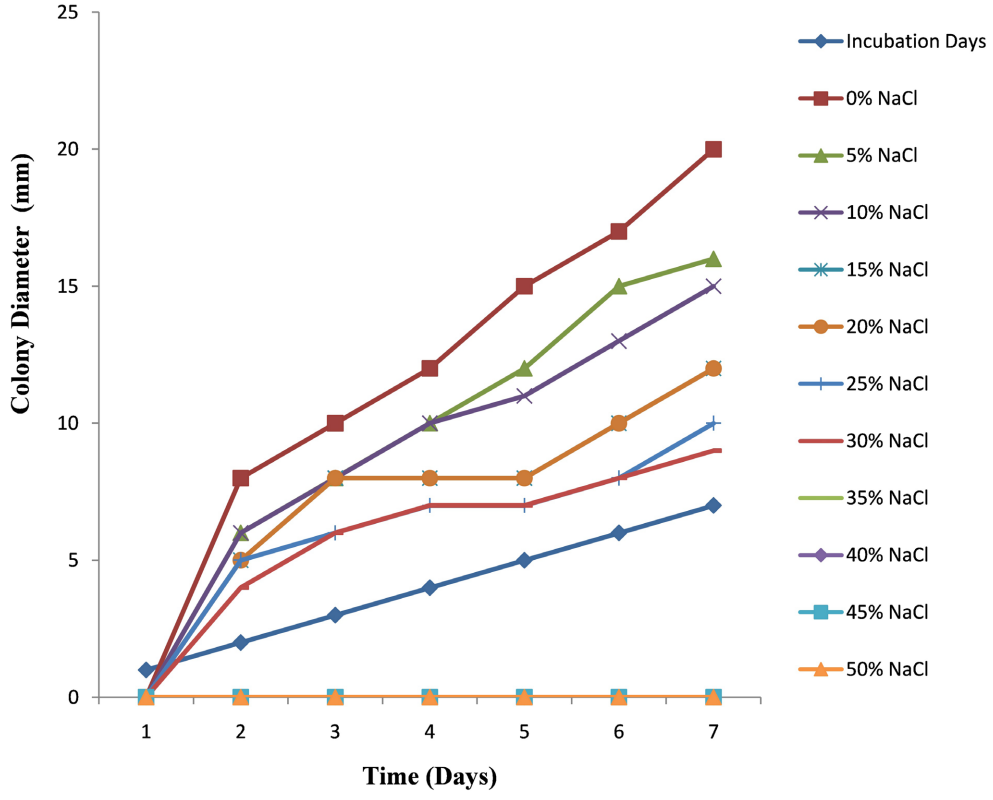

Figure 7. Halotolerance curve of *Trichoderma* sp.

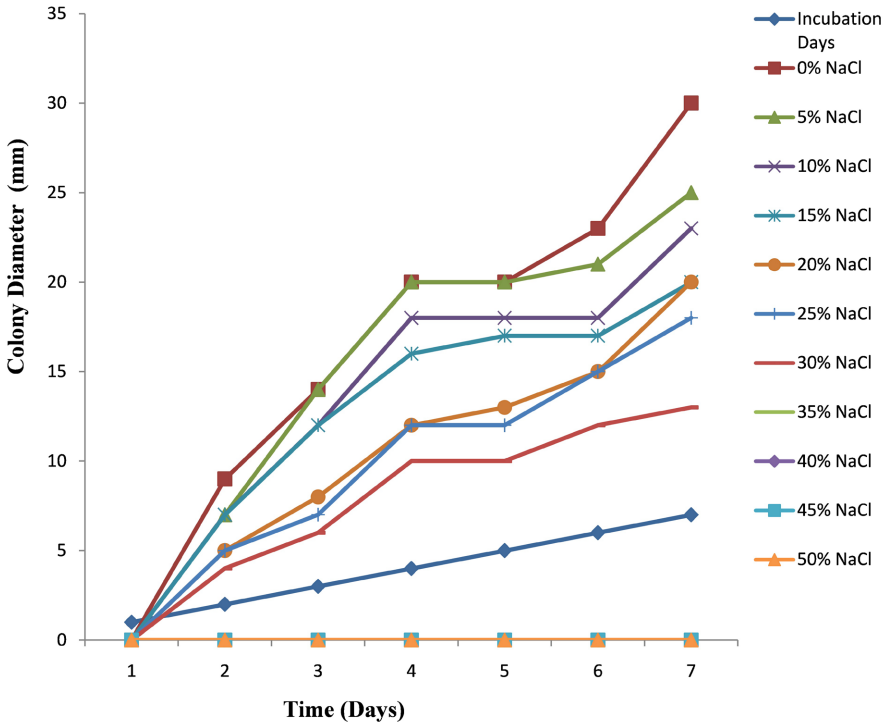

Figure 8. Halotolerance curve of *Mucor* sp.

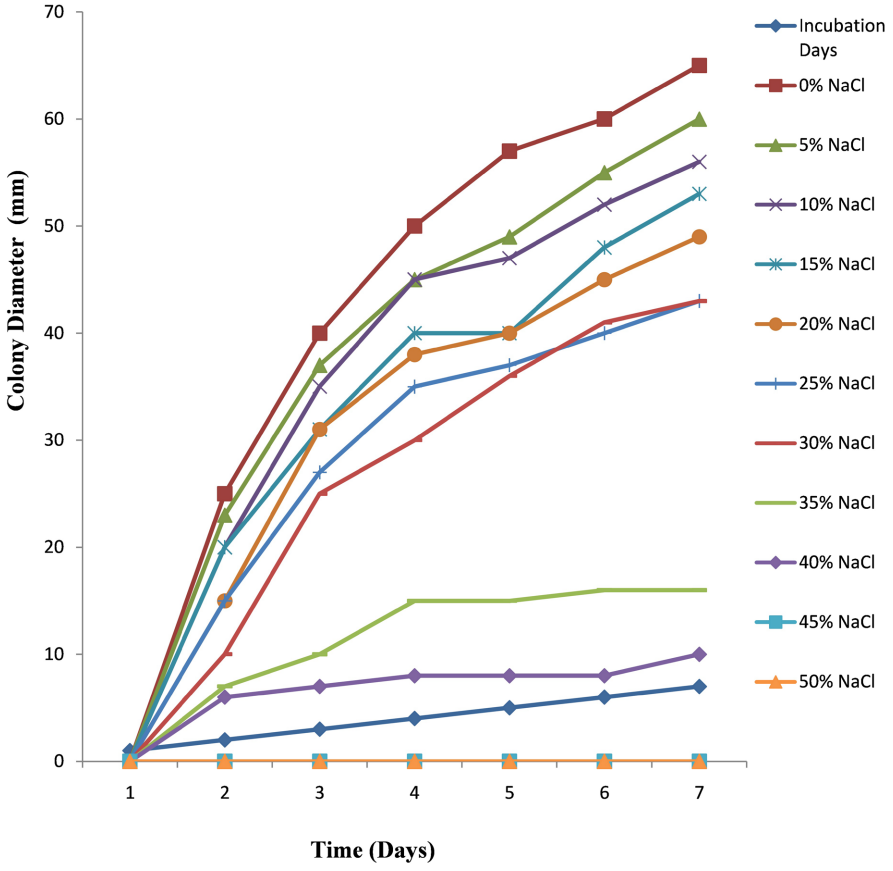

Figure 9. Halotolerance curve of *Penicillium citrinum*.

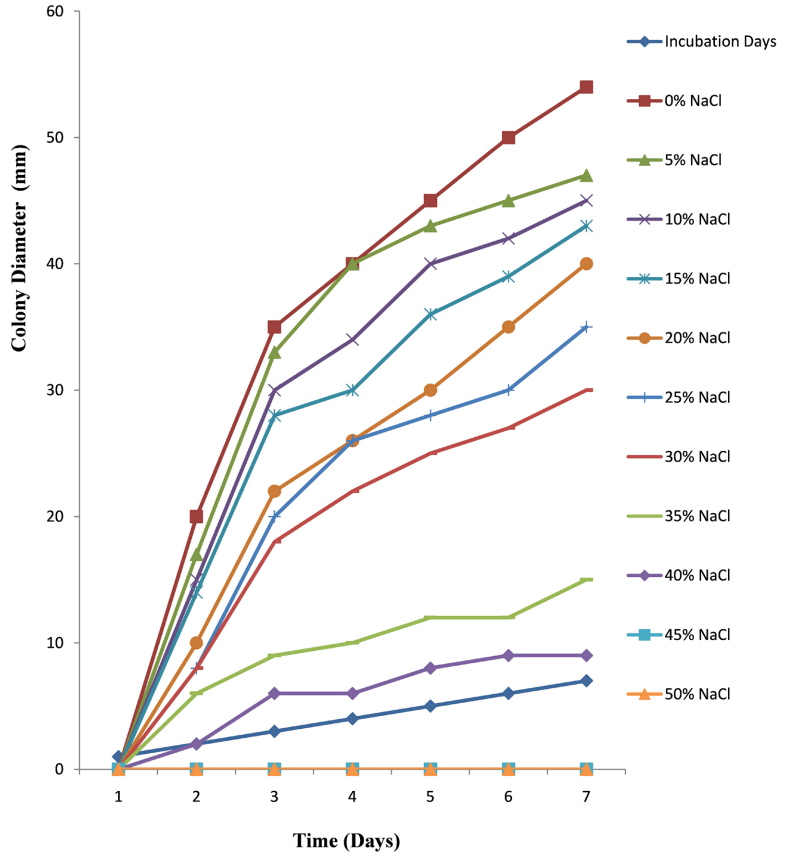

Figure 10. Halotolerance curve of *Aspergillus ochraceus*.

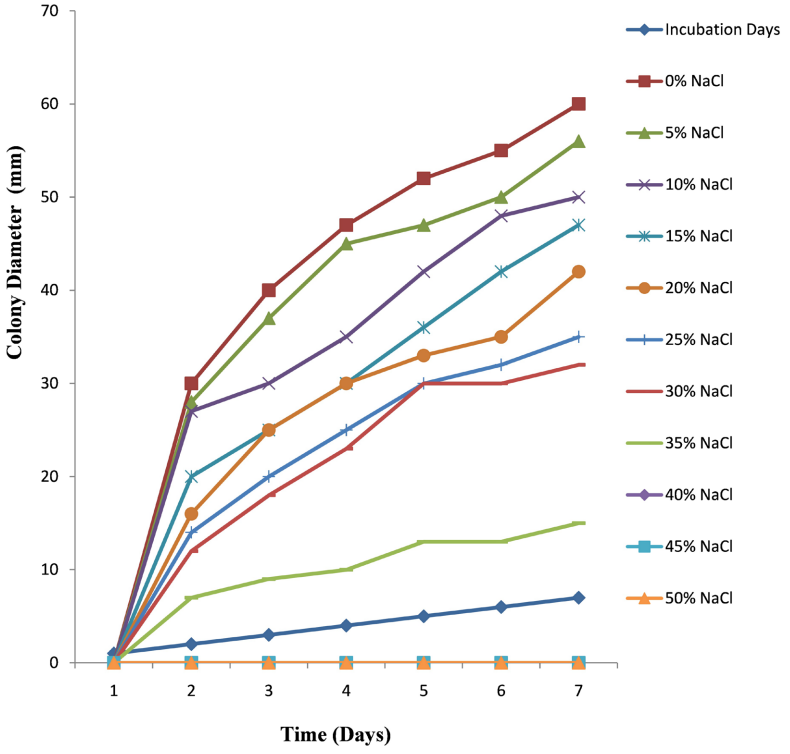

Figure 11. Halotolerance curve of *Aspergillus nomius*.

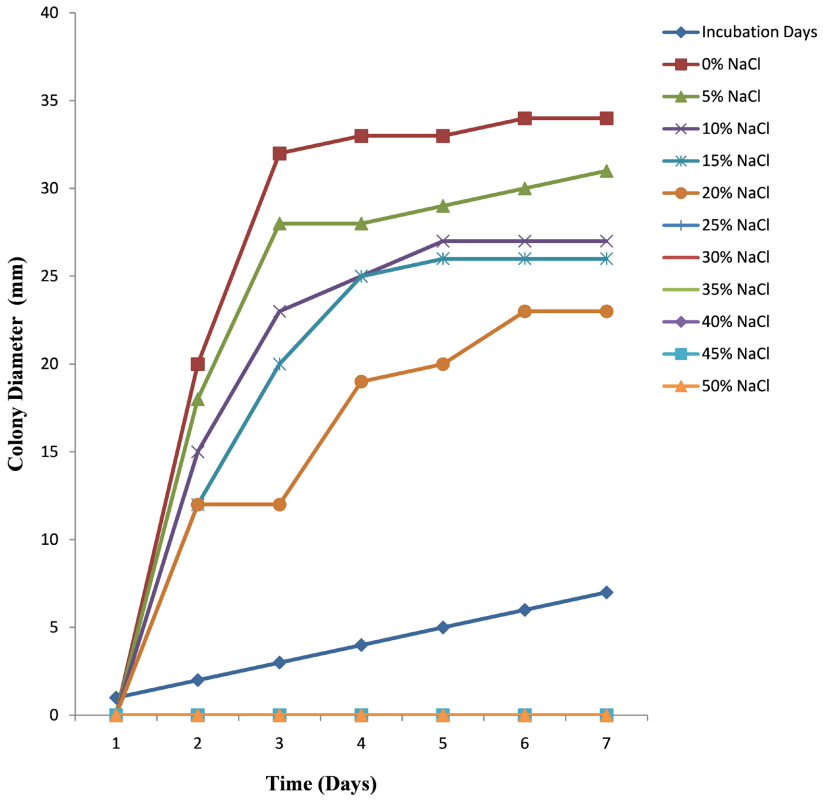

Figure 12. Halotolerance curve of *Penicillium* sp.

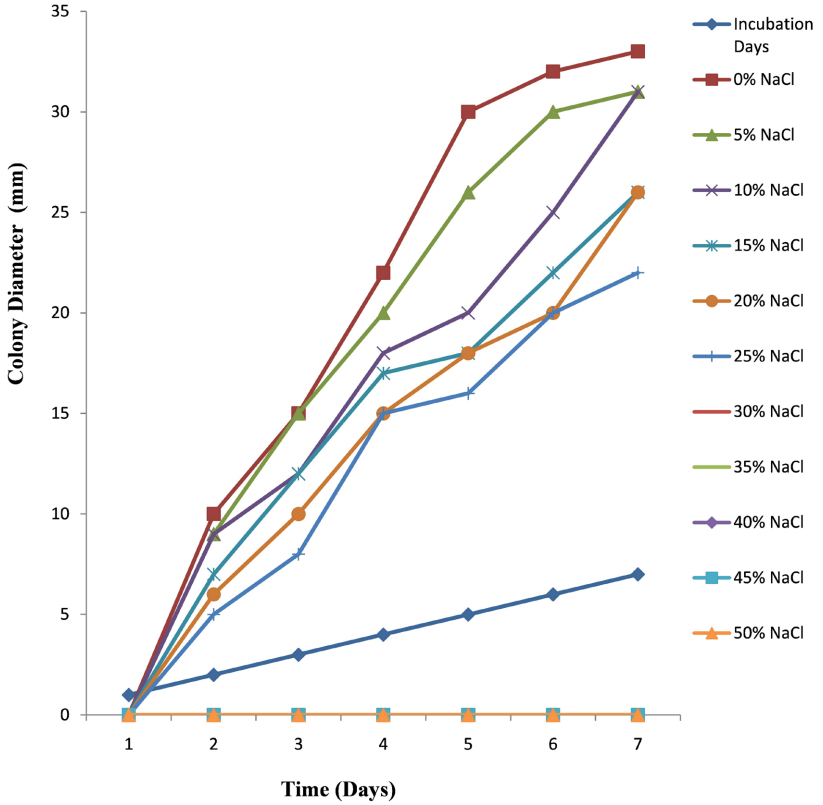

Figure 13. Halotolerance curve of *Alternaria* sp.

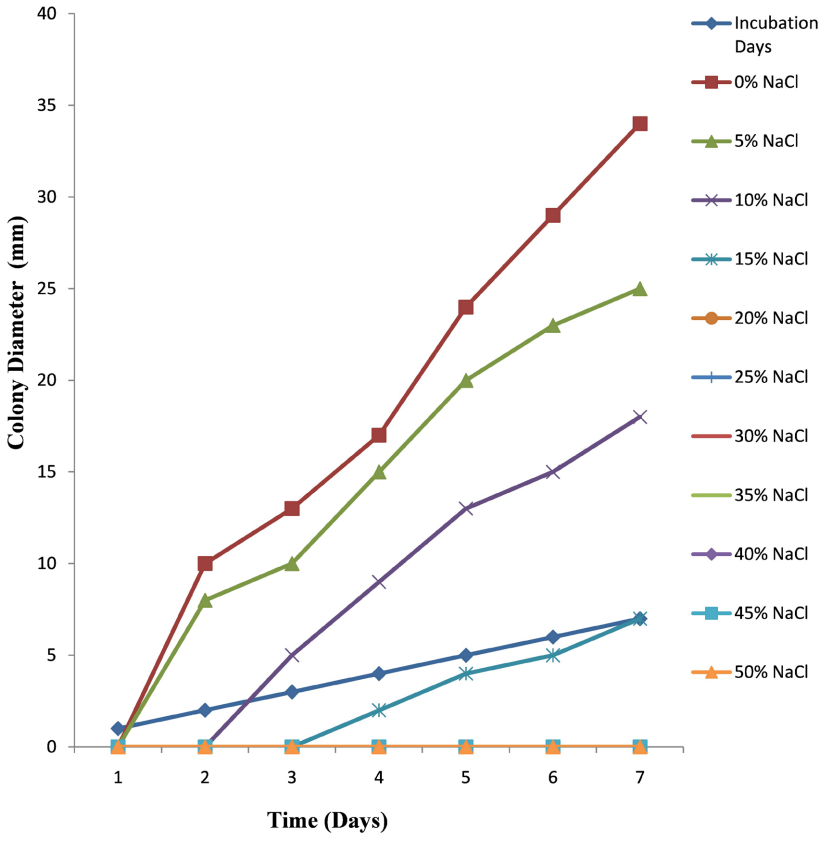

Figure 14. Halotolerance curve of *Cladosporium* sp.

4. Discussion

A little more than 20 years ago, scientists believed that hypersaline environments were predominantly inhabited by archaea, bacteria and a eukaryote—the alga named *Dunaliella salina*. But currently, several researchers such as [15] [38]-[42] isolated and reported a plethora of predominant eukaryotic fungal species like *Cladosporium*, different species of anamorphic *Aspergillus* and *Penicillium*, the teleomorphic *Emericella* and *Eurotium*, certain species of non-melanized yeasts represented by black, yeast-like hyphomycetes (mainly *Hortaea werneckii*, *Phaeotheca triangularis*, *Trimmatostroma salinum*, and *Aureobasidium pullulans*). Likewise, phylogenetically closely related species of *Cladosporium* belonging to the order Dothideales, and *Wallemia* sp from different hypersaline waters of the world. Findings from this work corroborated these initial published findings by isolating diverse halophilic fungi from Uburu and Okposi salt lakes (**Table 1** and **Table 2**). This present study carried out on Uburu and Okposi salt lake spanned throught out the 4 quarters and two seasons of a year, giving rise to a total of 14 different fungal isolates with varying halotolerance profiles *viz*: *Aspergillus sydowi* (28 mm at 30%), *Aspergillus flavipes* (13 mm at 40%), *Alternaria* sp. (22 mm at 25%), *Aspergillus ochraceus* (9 mm at 40%), *Microsphaeropsis arundinis* (12 mm at 35%), *Mucor* sp. (13 mm at 30%), *Aureobasidium* sp. (12 mm at 30%), *Penicillium citrinum* (10 mm at 40%), *Penicillium janthinellum* (26 mm at 30%),

Trichoderma sp. (9 mm at 30%), *Aspergillus* sp. (18 mm at 25%), *Penicillium* sp. (20 mm at 20%), *Aspergillus nomius* (15 mm at 35%), and *Cladosporium* sp. (7 mm at 15%) (**Figures 1-14**). The seasonal study was also divided into rainy season (April-September) and dry season (October-March). *Penicillium* and *Aspergillus* species were predominant throughout the study period in both lakes. However, the top 7 extreme halophilic fungi—*Microsphaeropsis arundinis*, *Aspergillus flavipes*, *Aspergillus ochraceus*, *Aspergillus nomius*, *Penicillium citrinum*, *Aspergillus sydowi* and *Penicillium janthinellum* were characterized and identified phenotypically and genotypically; while the rest were characterized only phenotypically as seen in **Table 1** and **Table 2**. Twelve isolates from the fourteen total isolates were classified as extremely halophilic, whereas one was a borderline extreme halophile, and the remaining isolate was a moderate halophile (**Table 3**). Plemenitaš and Gunde-Cimerman [43] stated that fungi isolated from hypersaline habitats of 1.7 M (~10%) salt concentration and can grow *in vitro* at or above 3 M (~18%) salt concentration should be considered as a halophilic fungus, and this served as the basis of the classification reported on **Table 3**. All the organisms isolated in this study tolerated this salinity range, and some of them exceeded the salinity range as well, and no slight halophile was isolated in this work. Fungal salt tolerance classification findings from this work partly correspond with the work of Mansour [44] who examined sandstone samples from the Medamoud, Egypt, and halophilic fungi (*Aspergillus nidulans*, *Aureobasidium pullulans* and *Cladosporium sphaerospermum*) from a local culture bank. Mansour estimated salinity tolerance by growing these fungi at 0% to 25% of NaCl concentration supplemented on potato dextrose agar and broth and opined that 5% of NaCl was the best suited growth supplement for halophilic fungi; and halophiles showed better tolerance to salt as compared to the solid media. They also reported that, 25% of NaCl concentration was found to inhibit any fungal growth. In this research however, solid medium was used and salt concentrations above 15% inhibited *Cladosporium* sp., but *Aureobasidium* and other species of *Aspergillus* grew well above borderline extreme salt ranges of 9% - 23%, as opposed to the findings of Mansour [44], as seen in **Figures 1-14**. The fact that no slight halophilic fungus was isolated in this work is also agrees with the reports of [9] [37] [45], who opined that microorganisms belonging to the slight halophiles are mostly marine bacteria such as *Vibrio*. The most halophilic fungi that was isolated in this study was *Aspergillus flavipes* which had a colony diameter of 13 mm at 40% salt concentration; hence it was used for further studies namely enzymatic and biodegradation studies which is not covered in the scope of this present publication. Enzyme complexes expressed by *A. flavipes* made it a useful biological agent in cancer research as an anti-tumor agent on the basis of its cytochalasin production [46]. This further shows the biotech and medical applications of this very important halophilic fungus. Other halophilic fungi have been reported to express enzyme complexes useful in textile manufacturing, detergent and surfactant production, bioremediation of industrial waste water contaminated with metal ions, agricultural waste water bioremediation, and also soy sauce production [47]. In contrast, halophilic archaea

and bacterium are not left out in their biotechnology usefulness. *Halobacterium salinarium* and *Haloferax volcanii* have been useful in biosensor technology for environmental toxicity monitoring studies [48]. *Bacillus megaterium* and *Halobacillus* sp. have been useful as metal ion bioremediators through nanoparticle formation technology [48]. Citing these few examples, it could be said that salt lakes do not just contain lethality and toxicity only, but also contain well-adapted useful extremophiles that serve the good interest of man and his environment.

5. Conclusion

Salt lakes are abundant, widely distributed geographically, and play a vital role in inland aquatic ecosystems worldwide. Their aesthetic, cultural, economic, recreational, scientific, conservation, and ecological values make them significant natural resources. Their microbiota composition uniquely distinguishes them from other aquatic ecosystems. Salt lakes develop as the termini of inland drainage basins where hydrological inputs and outputs are balanced. These conditions occur in arid and semi-arid regions (approximately one-third of the total world land area). Many human activities threaten or have already impacted salt lakes, especially surface inflow diversions, salinization and other catchment activities, mining, pollution, biological introduction, and anthropogenically induced climatic and atmospheric changes. In spite of these facts, Uburu and Okposi salt lakes have been less studied hitherto. In fact, there is paucity of research and data on the microbiological properties of both lakes, which has culminated in the disappearance of both lakes in the annals of salt lakes of the world, as have been noticed in numerous literatures and texts on saline lakes. This study presented seasonal fungal analysis of water samples obtained from Uburu and Okposi salt lakes, with subsequent identification using ITS rDNA sequencing (Macrogen, South Korea). The results revealed representatives of moderately to extremely halophilic fungi within both lakes. No slight halophile was isolated, out of the 14 fungi isolated. These fungi have abilities to adapt to a multi-extreme environment owing to the near-neutral to moderate alkaline and high salinity conditions of both lakes, as evidenced in the halotolerance curves of the recovered isolates. Culture dependent studies in future will help us unravel the survival mechanisms used by these polyextremophilic fungi.

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Conflicts of Interest

The authors declare no conflict of interest.

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