Microbes in deionized and tap water: Implications for maintenance of laboratory water production system

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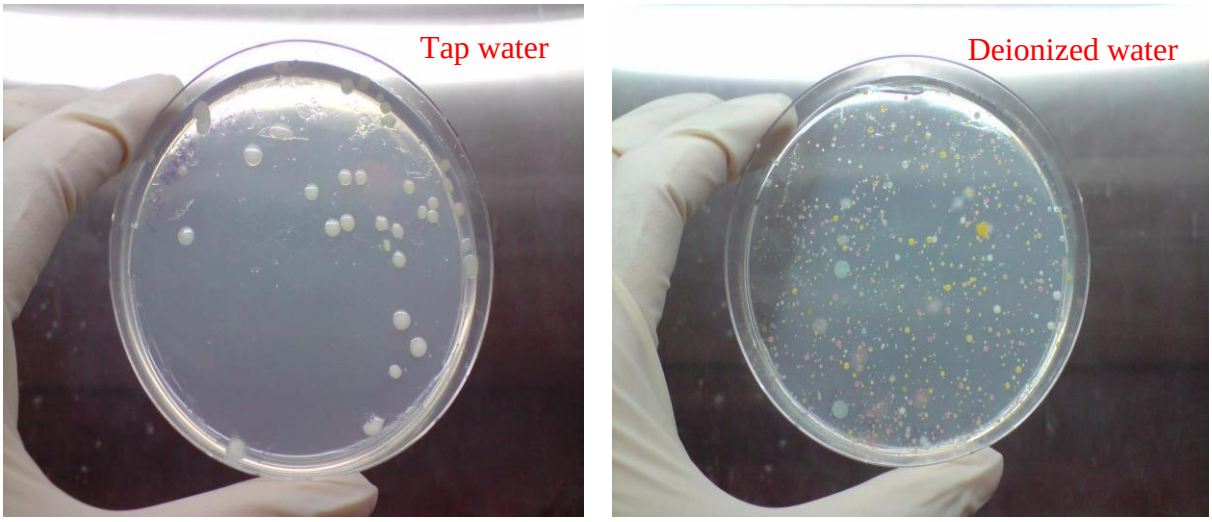
Graphical abstract



Short description: Microbes were recovered from deionized and tap water in significant numbers that calls for concern for both deionized and tap water quality. Specifically, greater diversity and relative abundance of microbes were recovered from deionized water compared to tap water on formulated colourless agar (with 0.1 g/L yeast extract) at 30 ^{o}C after multiple days of incubation. Possible removal of monochloramine residual disinfectant from tap water via ion exchange resins in deionized water production system might account for the greater recovery of microbes from deionized water compared to tap water. Close proximity of microbes further highlighted that possible exchange of metabolites and signaling molecules might facilitate the growth of more microbial species through a network of collaborative or neutral relationships. This suggested that antagonistic relationships were less prevalent in the consortium of microbes that constituted the microbial flora of deionized and tap water. Furthermore, keystone species could have enabled the growth of neighbouring microbial cells embedded in the agar matrix through the secretion of needed metabolites and signaling molecules, which generated zones with high colony density of microbes as well as others devoid of microbes due to absence of keystone species.

Abstract

Microbes, with their vast metabolic capabilities and great adaptability, occupy almost every conceivable ecological niche on Earth. Thus, could they survive in oligotrophic deionized (DI) water? Observations of white cauliflower-like lumps and black specks in salt solutions after

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months of storage in plastic bottles suggested a microbial origin for the "contaminants". Growth experiments was conducted to profile the microbial diversity of fresh DI water, produced on a just-in-time basis by a filter cum ion exchange system with tap water as feed. Inoculation of DI water on R2A agar and a formulated colourless agar followed by multi-day aerobic incubation revealed the presence of a large variety of microbes with differing pigmentations, growth rates, colony sizes and morphologies. Additionally, greater abundance and diversity of microorganisms was recovered at 30 ^{o}C compared to 25 and 37 ^{o}C; most probably due to adaptation of microbes to tropical ambient water temperatures of 25 to 30 ^{o}C. Comparative experiments with tap water as inoculum recovered a significantly smaller number and diversity of microorganisms; thus, suggesting that monochloramine residual disinfectant in tap water was effective in inhibiting cell viability. In contrast, possible removal of monochloramine by adsorption onto ion exchange resins of the DI water production system might explain the observed greater diversity and abundance of viable microbes in DI water. More importantly, greater diversity and abundance of microbes from tap water were recovered on R2A agar compared to formulated colourless agar, which suggested that chelating compounds in R2A agar could have complexed monochloramine and reduced its toxicity towards microbes. Similar chelating compounds were unlikely to be present in the formulated colourless agar. Finally, keystone species secreting signaling molecules and metabolites could induce the growth of neighbouring cells embedded in the agar matrix. This explained the presence of large clear zones devoid of colonies where there was no keystone species. Additionally, close proximity of colonies on agar suggested that cooperative and neutral relationships guided by exchange of metabolite and signaling molecules might be more prevalent compared to antagonistic relationships in which inhibitory compounds were used. Collectively, this study confirmed the presence of microbes in fresh DI water and tap water. Propensity of microbes in forming biofilm on various surfaces suggested that intermittent flow in just-in-time DI water production provided opportunities for cell attachment and biofilm formation during water stagnation, and subsequent dislodgement and resuspension of cells upon water flow. Thus, regular maintenance and cleaning of the production system should help reduce DI water's microorganism load.

Keywords**:** viable but not cultivable (VBNC), biofilm, microbial ecology, tap water, deionized water, chlorine residual, nutrient poor, monochloramine, disinfection, microbial flora,

Subject areas: microbiology, ecology, biodiversity, biochemistry, freshwater biology,

Significance of the work

Microbes varied in physiology and metabolic preferences and occupy almost every ecological niche on Earth. Thus, it would not be a surprise that microbes were found in deionized water and tap water. This study confirmed the presence of large diversity and numbers of microorganisms in deionized water. Microbes of differing pigmentations, colony size and morphology were also recovered from tap water. Adsorption and removal of residual disinfectant, monochloramine, by ion exchange resins of the deionized water production system was postulated to account for the greater recovery of microorganisms in deionized water compared to tap water. Greater diversity and numbers of microbes were also recovered at 30 ^{o}C compared to 25 and 37 ^{o}C, which indicated that microbes in tap and deionized water had adapted to tropical ambient temperatures of ~30 ^{o}C. Close proximity of many recovered colonies from deionized and tap water revealed that there was a greater preponderance of neutral and collaborative relationships compared to antagonistic ones

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amongst microbes. Specifically, secretion and exchange of metabolites and signaling molecules could have enabled the recovery of many colonies. Finally, keystone species that secrete useful metabolites and signaling molecules needed by other microbes could have played a critical role in enabling the recovery of more microbes on both R2A and a formulated colourless agar. Areas on agar without such keystone species would appear as clear zones devoid of colonies due to the lack of secreted metabolites or signaling molecules that facilitated the recovery of microorganisms.

Highlights

1) Multiple types of microbial colonies at high colony density were recovered from deionized and tap water on R2A and a formulated colourless agar medium.

2) Greater diversity and relative abundance of microbes was recovered from deionized water compared to tap water, due probably to the removal of monochloramine residual disinfectant through adsorption on ion exchange resins of the deionized water production system.

3) Compared to incubation at 25 and 37 °C, more colonies of different types were recovered on both R2A and formulated colourless agar at 30 °C, which suggested that microbes in tap water had adapted to tropical ambient water temperature of ~30 °C.

4) R2A agar was also better at recovering microbes from tap water relative to formulated colourless agar, due probably to the presence of chelating compounds in R2A agar that sequestered monochloramine disinfectant and rendered it less toxic to microbes.

5) Close proximity of colonies to each other suggested that cooperative or neutral relationships forged by an exchange of metabolites or signalling molecules might be dominant over antagonistic ones. More importantly, exchange of signalling molecules and metabolites between microbes could enable the growth of species previously unable to grow on either agar media.

6) Keystone species might secrete metabolites and signalling molecules that enabled the growth of other close-by microbes embedded in the agar matrix; thereby, creating zones with high density of microbes where the keystone species is present, as well as others devoid of microbes without the keystone species.

7) Recovery of microbes from deionized and tap water at 37 °C suggested potential human pathogens in deionized and tap water biofilms.

Introduction

Microorganisms occupy every conceivable niche on Earth, whether in deep ocean hydrothermal vents or within the Earth crust.[1 2 3 4 5] Hence, microbes, in aggregate, possess versatile metabolism and adaptability for coping with various nutritional conditions and environmental stressors on Earth. Individual species of microorganism, however, do not possess all requisite metabolic pathways for surviving various environmental conditions.[6] Thus, differences in nutritional conditions and environmental stressors at different locales could account

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for differing metabolic characteristics of microbes present in the habitat. For example, microbes able to survive in drinking water possess the ability to harness small amounts of nutrients (i.e., oligotrophic environment) for survival and growth, while bacteria with a high energy metabolism requiring constant infusion of nutrients for survival would likely not be able to survive in potable water.

Observations of cauliflower-like lumps and black specks in prepared salts solutions contained in plastic bottles suggested deionized water as a possible source of contaminants. As a low nutrient growth environment, deionized water is not conducive for microbial growth; however, microbes could remain viable even though they are not in the growth state characterized by rapid cell division. Many approaches and agar media have been used in inducing the growth of microorganisms from tap water or other water matrixes with low nutrient content, but they generally could not recover significant numbers of microbes thought to be present in the water.[7] By mimicking the natural low nutritional environment of various environmental matrixes such as tap water, R2A agar is the gold standard agar medium for the cultivation of microorganisms from oligotrophic environments.[8] Specifically, slow growth rates in a low nutrient environment provides multiple microbial species with an opportunity to grow given that access to nutrients was not constrained to species with fast growth rates. Results indicated that R2A agar could recover significantly more species of microorganisms from tap water compared to other agar media. This motivated the formulation of a similar low nutrient agar medium, which in being colourless, help improve the optical transparency of the agar that improves the identification of small colonies.[9]

Hence, using R2A agar and a formulated colourless agar with deionized and tap water as inoculum, growth experiments via spread plate inoculation was performed at 25, 30 and 37 ^{o}C. Recovery of a large variety of microorganisms of different pigmentation, morphology, and growth rates revealed that a diverse microbial flora existed in deionized water, due possibly to the removal of the residual disinfectant monochloramine from tap water by ion exchange resins of the deionized water production system. Tap water, in comparison, harboured fewer number and types of viable microbes relative to deionized water; thereby, indicating that monochloramine was effective in inhibiting the growth of microbes in tap water. Thus, removal of monochloramine by adsorption on ion exchange resins in the deionized water production system likely removed an environmental stressor inhibiting the growth of microbes in tap water. Furthermore, possible poor maintenance of the filter cum ion exchange column critical to production of deionized water could provide a concentrator of microorganisms through the growth of biofilms on the surfaces of the filter membrane of the deionized water production system.

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Materials and Methods

Materials

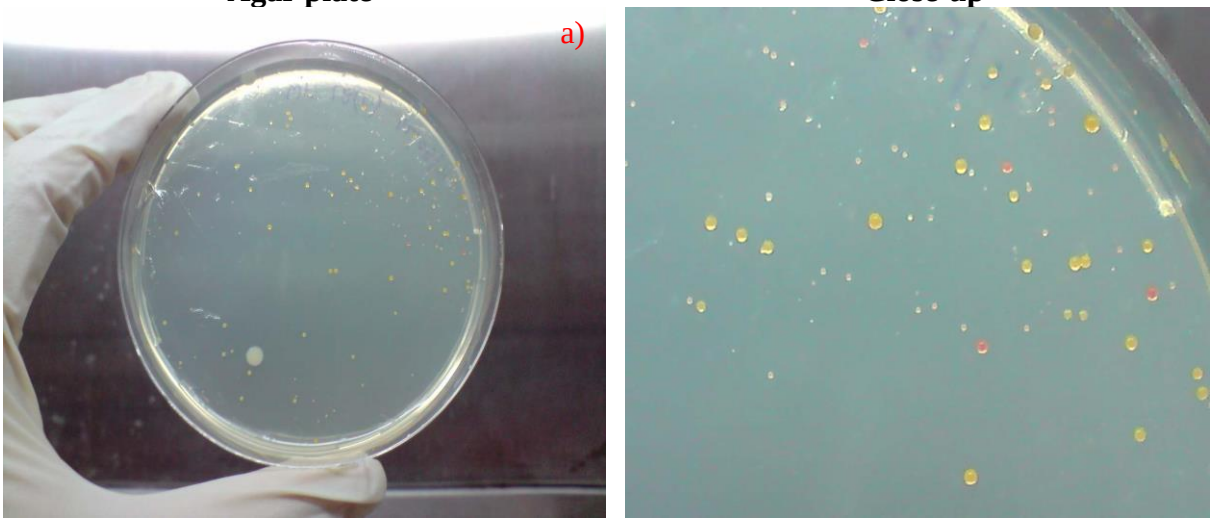
R2A and Tryptic Soy agar were purchased from Merck and used as is. Composition of R2A agar was [g/L]: yeast extract, 0.5; Proteose Peptone, 0.5; Casein hydrolysate, 0.5; Glucose, 0.5; Starch soluble, 0.5; Sodium pyruvate, 0.3; K_2HPO_4, 0.3; $MgSO_4$, 0.024; Agar, 15.0. Composition of formulated colourless agar was [g/L]: D-Glucose, 2.0; NH_4Cl, 0.5; K_2HPO_4, 0.5; KH_2PO_4, 0.1; NaCl, 0.5; $MgSO_4.7H_2O$, 1.0; Yeast extract, 1.0; Agar, 15.0. Composition of LB Lennox agar was [g/L]: Tryptone, 10.0; Yeast extract, 5.0; NaCl, 5.0; Agar, 15.0. Composition of M9 agar was [g/L]: D-Glucose, 4.0; NH_4Cl, 1.0; Na_2HPO_4, 6.78; NaH_2PO_4, 3.0; NaCl, 0.5; Agar, 15.0. Composition of Tryptic Soy Agar was [g/L]: Pancreatic digest of casein, 15.0; Papaic digest of soya bean, 5.0; NaCl, 5.0; Agar, 15.0.

Growth of microbes on solid medium

Tap and deionized water were collected fresh from the tap and deionized water production system respectively after allowing the water to run for 5 minutes, and was contained in a pre-sterilized 15 ml polypropylene centrifuge tube. The deionized water production system was equipped with ELGA Stat cation and anion exchange resins system preceded by a filter membrane cartridge. 0.1 ml of tap or deionized water was used as inoculum for either R2A agar or formulated colourless agar and inoculated via the spread plate method. Inoculated plates were incubated aerobically in a temperature controlled incubator at the chosen temperature (Yih Der LM570D, Taiwan). Agar plates were observed daily and at suitable time points, photographs were taken of the agar plates in a Class II Biosafety cabinet. Three biological replicates were performed in each experiment.

Results and Discussion

Agar plate **Close-up**



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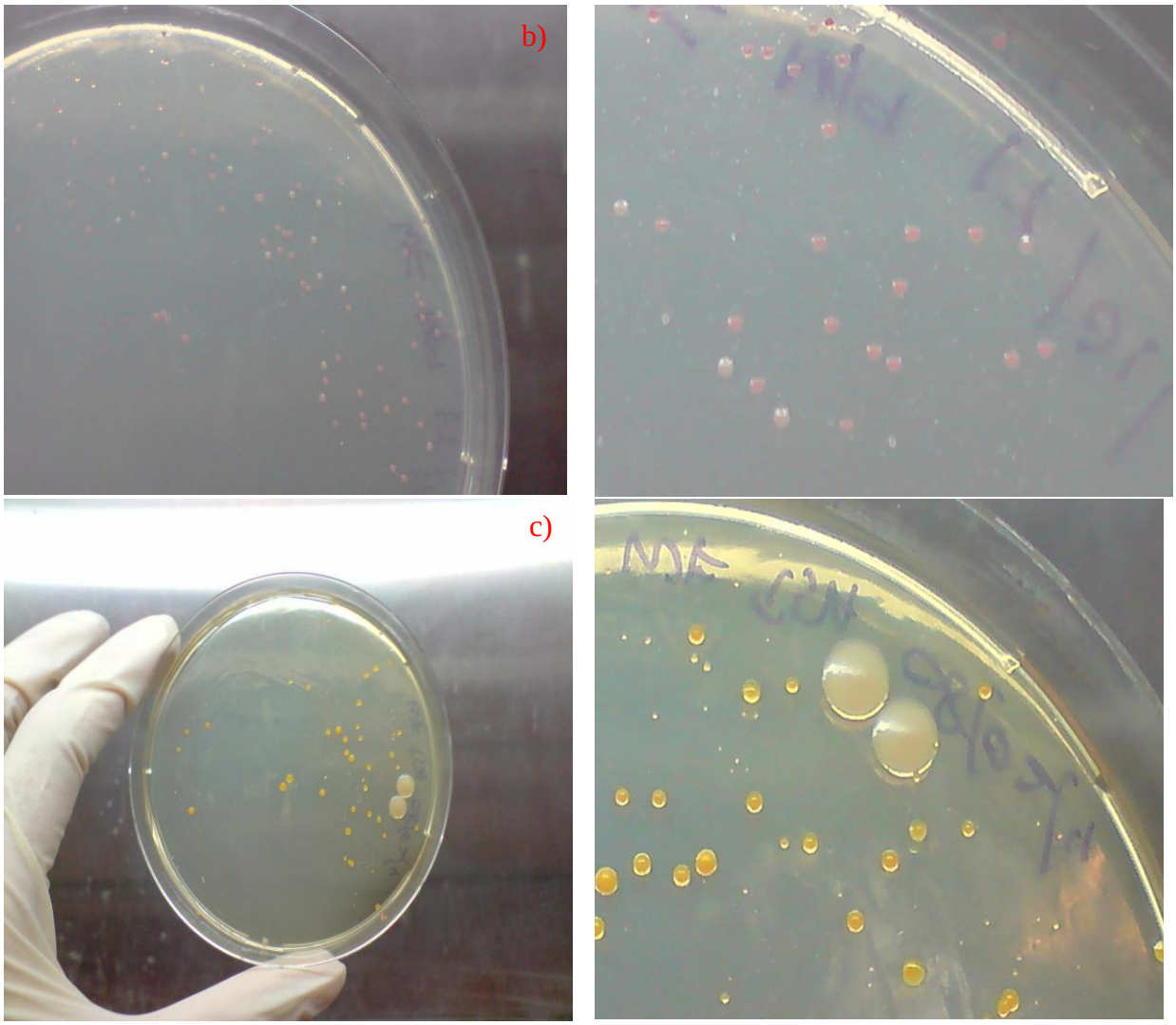

Figure 1: Growth of microorganisms from deionized water on various rich media at 30 ^{o}C incubation. a) LB Lennox medium after 2.5 days of incubation, b) M9 medium after 8 days of incubation, and c) Tryptic Soy Agar (TSA) agar after 8 days of incubation.

Microbes in oligotrophic environment such as the nutrient poor deionized water and tap water typically grow very slowly on recovery on agar media. Additionally, use of rich medium with high nutritional content typically hamper the recovery of many microbial species present in environmental matrixes such as freshwater due to the accelerated growth of a small subset of species able to utilize the abundant nutrients available. This, in turn, reduced nutrients available for growth of other species that grow more slowly. To understand the subset of microbes in deionized water able to grow in rich media such as LB Lennox, M9 and Tryptic Soy Agar (TSA), spread plate inoculation of deionized water inoculum was conducted on the above media and the agar plates incubated at 30 ^{o}C for multiple days. Experiment results revealed that relatively few microbial species could be cultivated on rich media such as LB Lennox agar, M9 agar and TSA agar (Figure 1). More importantly, microbial species cultivated were dominated by one or two

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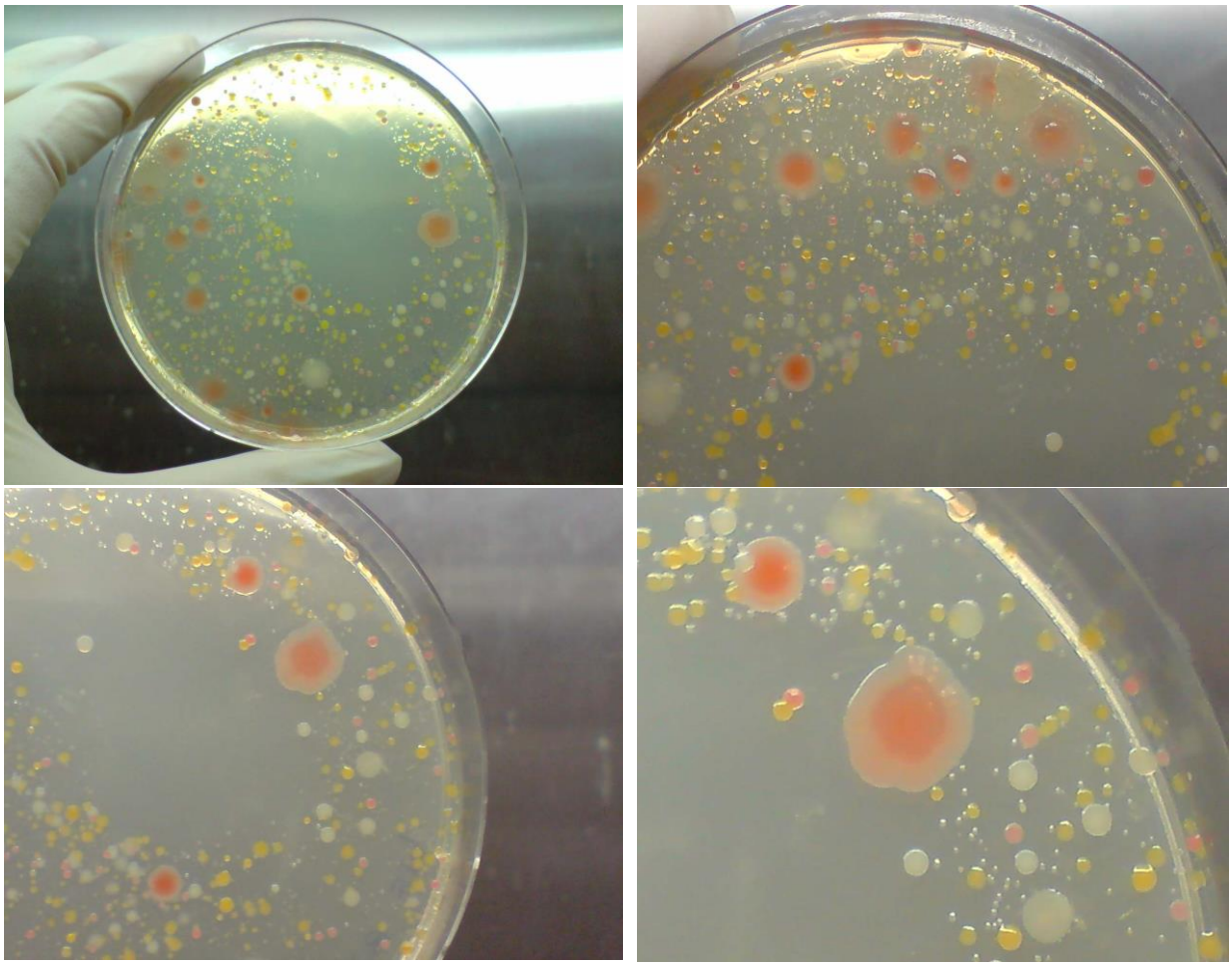
types of colonies on the agar used, which revealed that microbes adapted to a low nutritional environment could not adapt to a high nutrient environment due possibly to the presence of a metabolic sensory system able to determine the nutritional and energy state of the environment surrounding a microbe. Thus, the types of nutrients that form the basis for determining the nutritional state of the environment with respect to a microbe would be key to understanding the principles of medium design for enabling the cultivation of the broadest subset of microbes from environmental samples. Overall, results revealed that rich media such as LB Lennox, M9 and TSA could not be used in understanding the microbial flora of deionized water.



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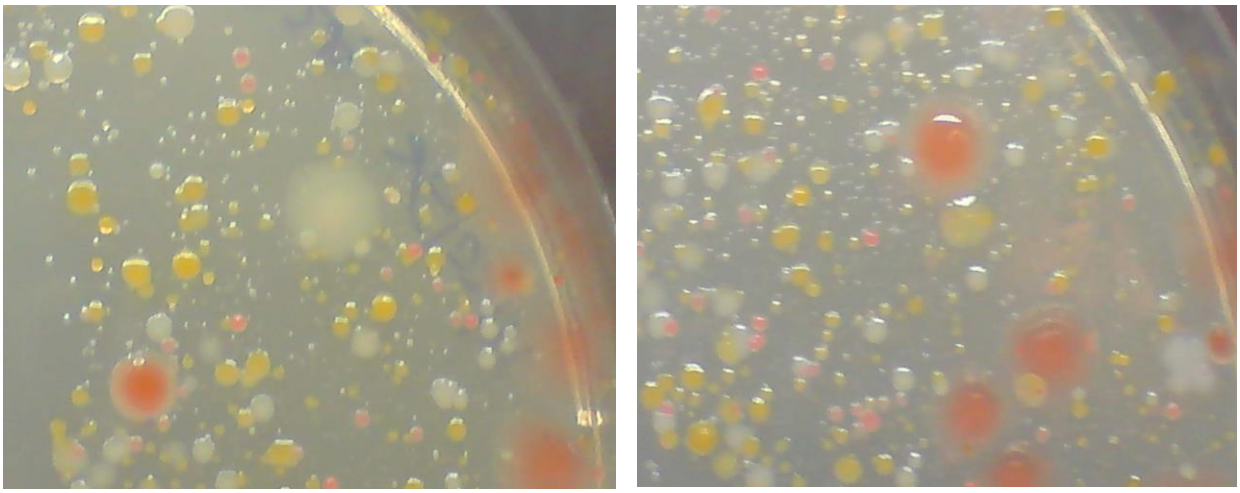

Figure 2: Growth of microbes in deionized water on R2A agar for 8 days at 30 ^{o}C. Photo of the agar plate followed by close-up pictures of the same plate.

Growth of microorganisms from deionized water on R2A agar (Figure 2) revealed that a large variety of microbes were present in deionized water and were viable. Specifically, colonies of different size, pigmentation and affinity to oxygen were observed on R2A agar plates at a high colony density, which indicated that growth inhbitory compounds were generally not secreted between the different microbial species. Specifically, small, round colonies were seen embedded within the agar matrix, which suggested that there were microaerophilic. Different types of colonies were seen in close proximity to each other; thereby, suggesting possible symbiotic relationships. For example, microbe A might secrete metabolites into the medium useful for a close-by microbe B; thereby, allowing microbe B to grow in the medium. Similarly, microbial cells could exchange signalling molecules between species.[10] [11] [12] Cast into a broader picture, the results suggested that a complex microbial consortium likely formed a biofilm on filter membrane of the deionized water production system. Cells were disloged from the biofilm on a regular basis due to shear stress of water flow. Additionally, adsorption of monochloramine disinfectant on ion exchange resins reduced the environmental stress on the microbes and facilitated their regain of viability when inoculated on R2A agar.

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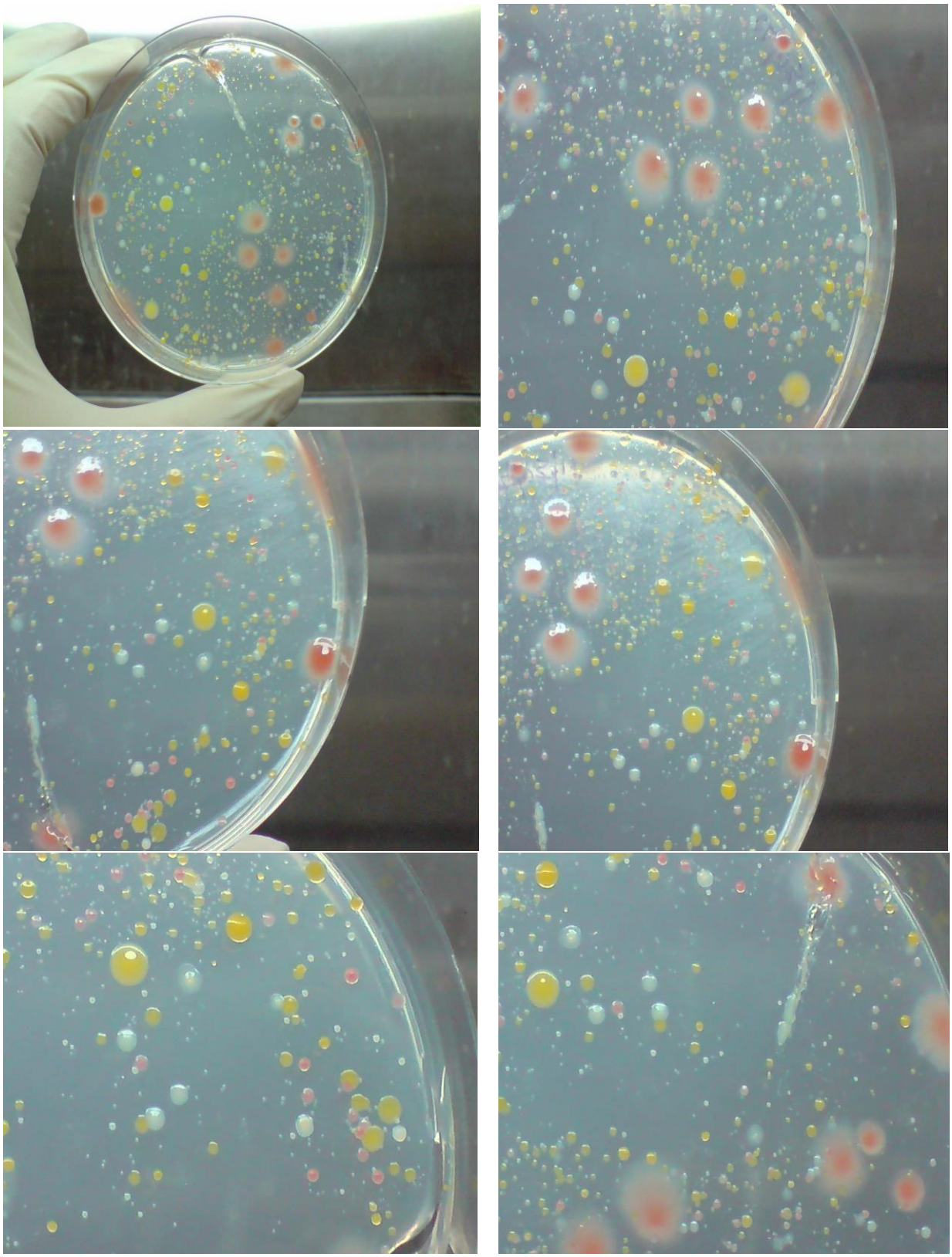

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Figure 3: Growth of microbes in deionized water on formulated colourless agar with 1 g/L of yeast extract at 30 ºC for 8 days. Photo of agar plate followed by close-up pictures of the same plate.

Similar growth experiments conducted using spread plate inoculation of deionized water inoculum on formulated colourless agar with 1 g/L of yeast extract recovered large diversity of microbes of different colony morphology, growth rates, pigmentation and sizes (Figure 3). Small, round colonies were also seen embedded within the agar matrix, which meant that they were likely microaerophilic. Ability to recover a wide variety of microorganisms meant that the formulated colourless agar did not harbour growth inihibitory compounds detrimental to growth. Given that the different colonies were in close proximity to each other, with some in close contact, growth inhibitory compounds such as antibiotic was likely not secreted by the microbes. This revealed that the microbial species had adapted to growth in close proximity to each other, where intercellular signalling could have formed the communication basis of a cooperative or neutral relationship instead of antagonistic ones.

Furthermore, recovery of the consortium of microbes on agar also revealed that a large variety of microbes likely constituted the microbial community in deionized water production system, which holds important implications to our understanding of microbial ecology of water. Specifically, could antagonistic microbes be induced to form neutral relationships in the face of severe environmental stressors such as the disinfectant monochloramine and low nutrients? From another perspective, it is interesting to note that there was no substantial clear circular zones surrounding individual colonies on both R2A and formulated colourless agar, which indicated that antibiotic secretion between different microbial species was absent. Absence of antibiotic warfare between microbial species provided a platform for the concurrent cultivation of many species on the same agar that aids in understanding the diversity of microbes present. It also speaks of the potential absence of major antagnoistic relationships between different microbes in deionized water that lead to an interesting question in microbial ecology: could survival and growth in close proximity in a biofilm environment change the inter-relationships between microbes from competitive to cooperation?

Monochloramine, when present, is a source of environmental stress for microbial survival given its known toxicity towards microbes as well as its ability at inhibiting their viability.[13] [14] [15] [16] [17] Hence, adsorption of monochloramine by ion exchange resins of the deionized water production system removed a significant source of environmental stress to the microbes; thereby, enabling their return to viability, which resulted in the recovery of large diversity of microbes in deionized water on both R2A and formulated colourless agar medium. Finally, given that monohloramine was most probably adsorbed by ion exchange resins, the high surface concentration of the disinfectant on the resins meant that biofilm would likely not form extensively on the resins' surfaces. Hence, microorganisms present in deionized water likely reside on the

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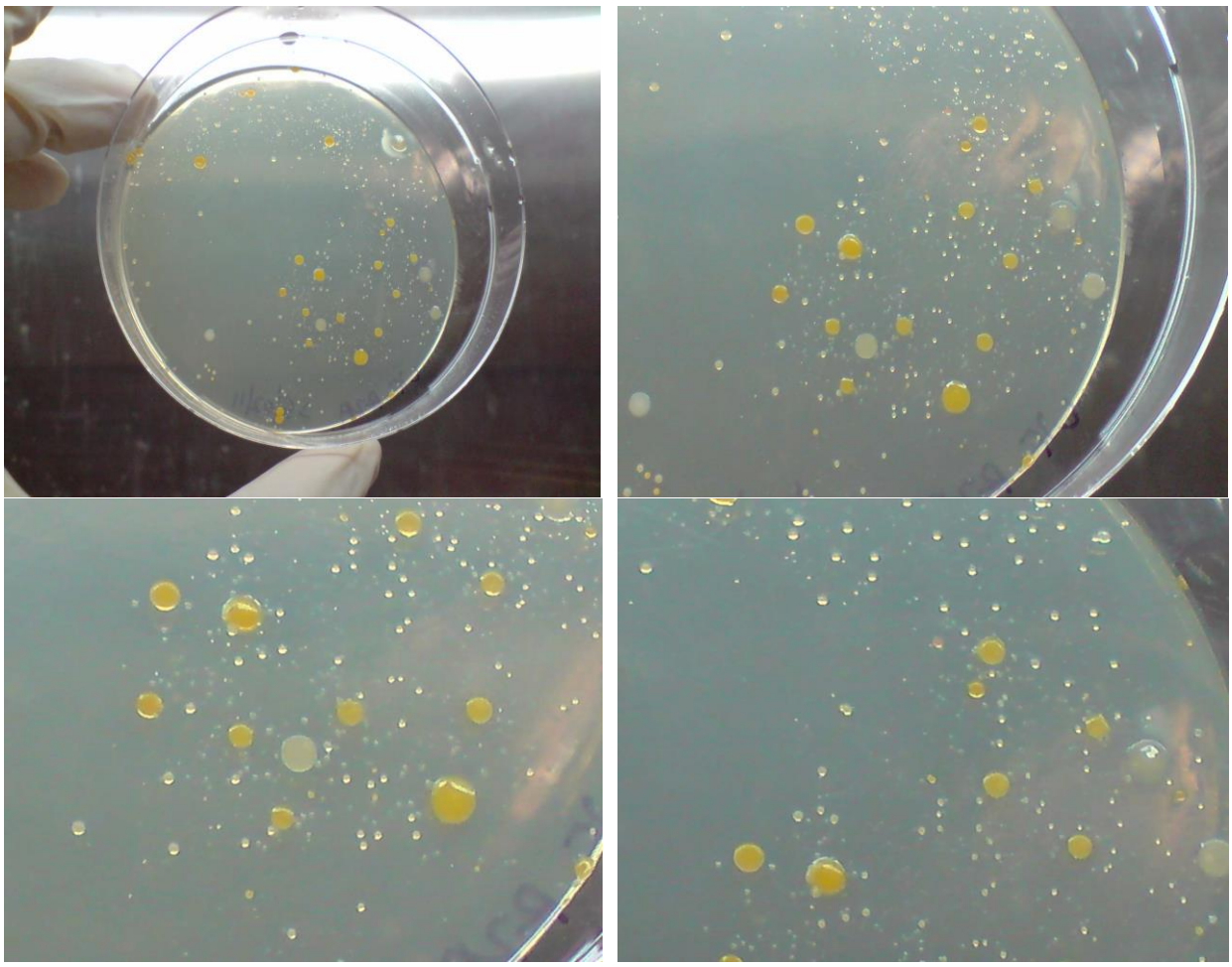
surfaces of the filter membrane that preceded the ion exchange system of the deionized water production system. Thus, observed large numbers and diverse types of colonies from deionized water could be due to a combination of relatively high microorganism concentration in tap water and the concentration of microbes on biofilm in the filter unit of the deionized water production system.



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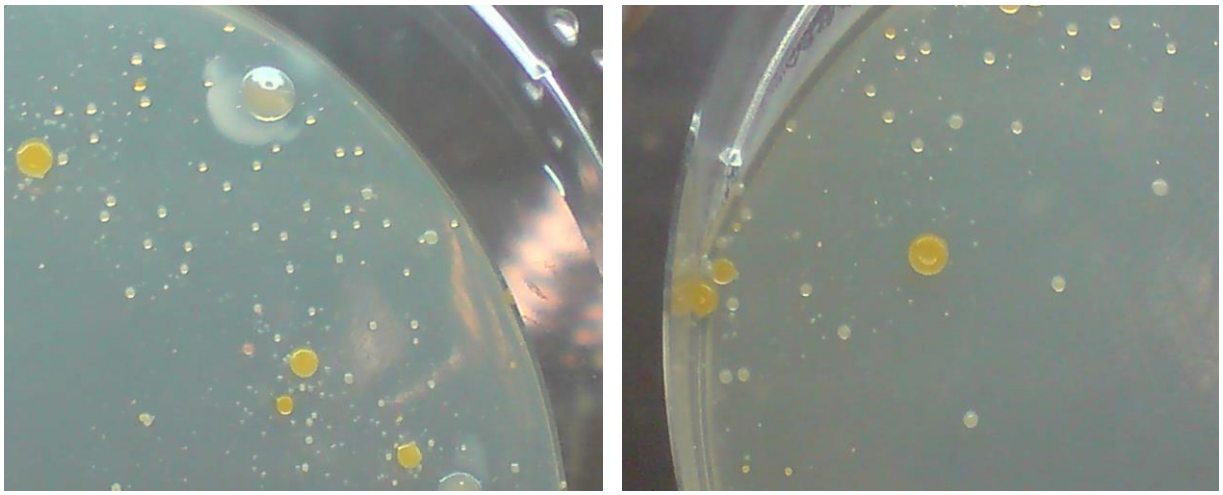

Figure 4: Growth of microorganisms from tap water on R2A agar. Incubation conditions: 30 ºC for 7 days. Photo of agar plate followed by close-up pictures of the same plate.

Comparative growth of microbes in tap water on R2A agar for understanding the provenance of the microbes in deionized water revealed significant reduction in numbers and types of colonies recovered (Figure 4). However, significant number of different types of microbes remain recoverable; thereby, indicating that the microbial load of tap water was likely high. With the presence of monochloramine as a residual disinfectant, microbial viability in tap water was likely significantly lower compared to deionized water, which explained the significant reduction in number of colonies recovered.

More importantly, given the close proximity of different types of colonies from tap water recovered on R2A agar, antagonistic relationships between microorganisms in tap water were likely to be few, which suggested that the drinking water biofilm[18 19 20 21] that existed in the drinking water distribution pipeline harbours species of microbes able to form synergistic and non-competitive relationships with each other. Hence, forced growth in defined community such as a drinking water biofilm might have engendered communications between different microbial species that reshape the community structure of the biofilm, lending a less competitive relationship to ones previously known to be competitive. Such modulation of microbes' behaviour, if shown to be true, would be a significant advance in our understanding of microbial cell-cell communication.

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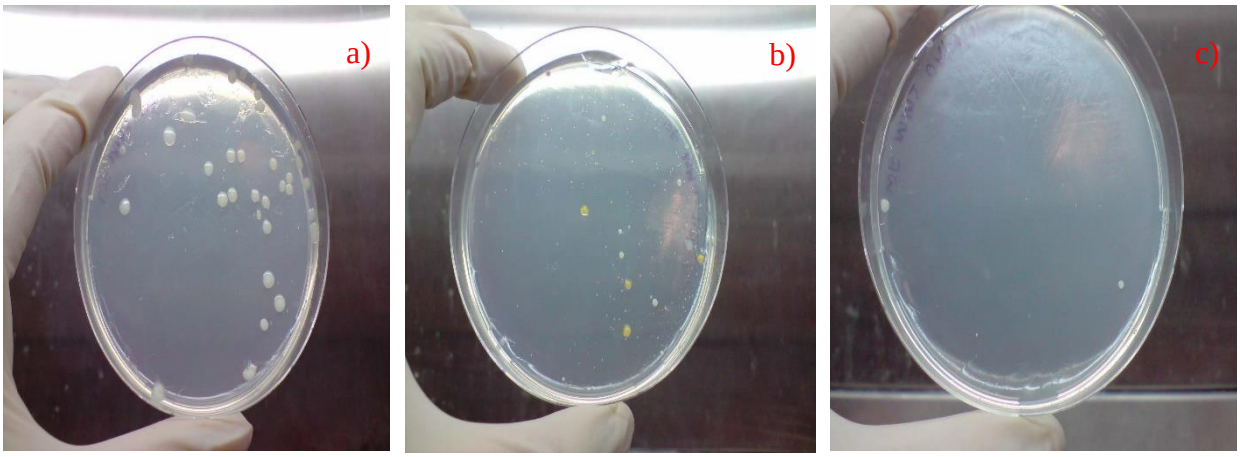

Figure 5: Cultivation of microbes in tap water on formulated colourless agar at 30 ºC with different concentrations of yeast extract supplementation: a) 0.1 g/L yeast extract (8 days of incubation), b) 0.5 g/L yeast extract (7 days of incubation), and c) 1.0 g/L yeast extract (6 days of incubation).

Experiments aimed at understanding the growth behaviour of microbes in tap water on formulated colourless agar at 30 ºC revealed a substantial reduction in the types and numbers of microbes that could be recovered on the agar medium (Figure 5). Specifically, only a few dominant types of microbes could be recovered. Hence, amount of yeast extract concentration in the colourless agar medium was varied for understanding if the amount of vitamins and growth factors supplemented for growth could affect the recovery rates of microbes in tap water. Results revealed that yeast extract of 1 g/L was not useful for recovering more types of microbes in tap water. On the other hand, a single dominant type of microbe of white round colony was recovered at yeast extract of 0.1 g/L. Finally, 0.5 g/L of yeast extract appeared to be the optimal concentration for inducing the growth of more types of microbes from tap water (Figure 6). Close proximity of colonies to each other revealed that cooperative and neutral relationships likely dominate over antagonistic ones. In addition, inhibitory compounds were likely not secreted by microbes which would have affected the growth of nearby colonies. Finally, metabolites and signalling molecules could have been exchanged between nearby colonies.

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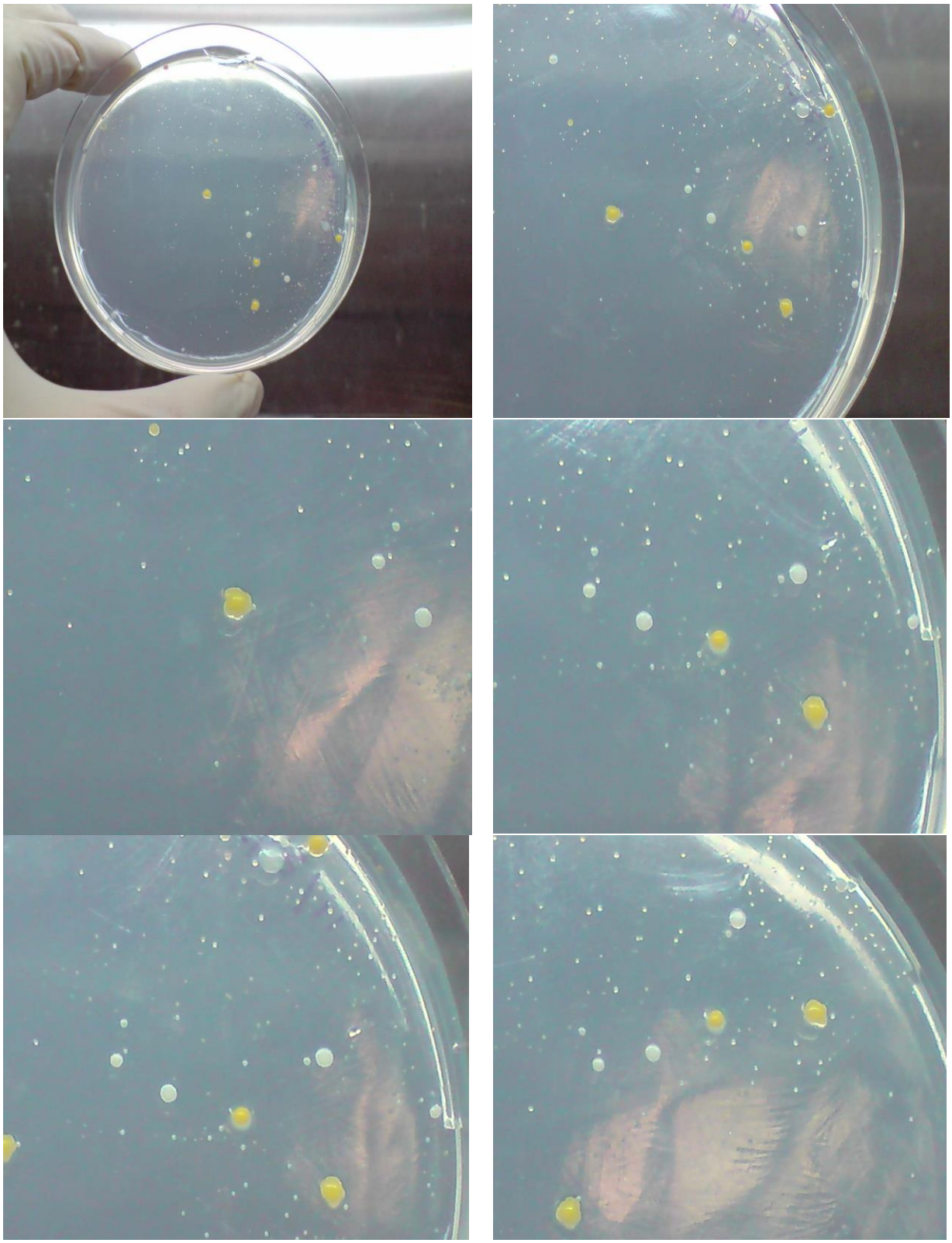

Figure 6: Growth of microorganisms in tap water on formulated colourless agar with 0.5 g/L of yeast extract supplementation at 30 ^{o}C for 7 days. Photo of agar plate followed by close-up pictures

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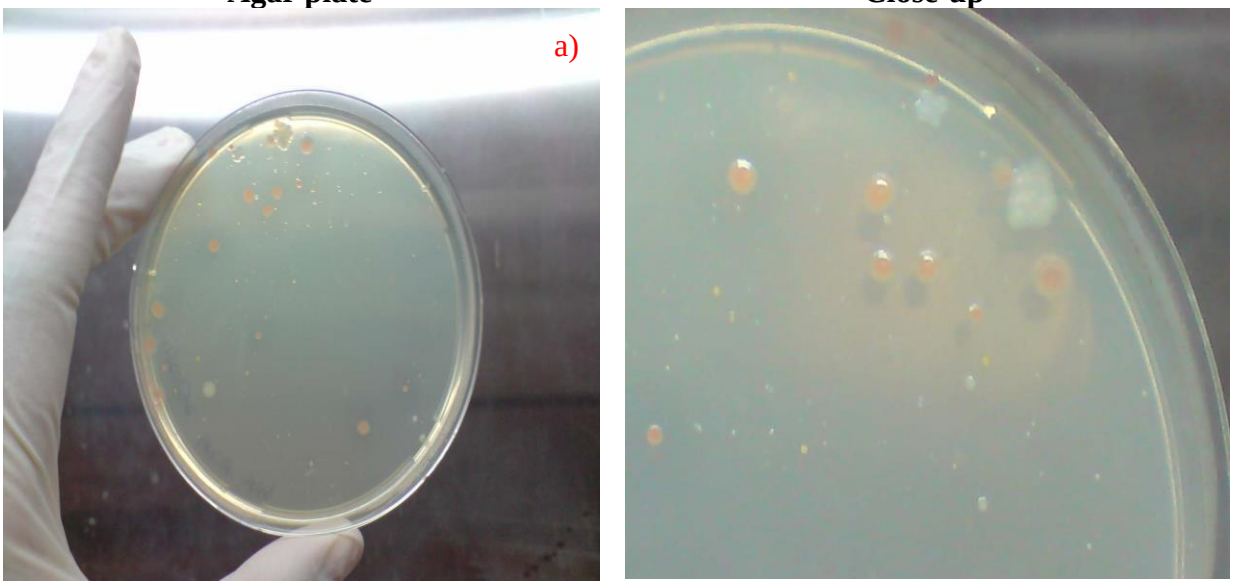
of the same plate revealed many small colonies could be recovered from tap water after extended incubation of a few days.

Overall, R2A agar was demonstrably able to recover more types and numbers of microbes from tap water compared to formulated colourless agar with different concentrations of yeast extract supplementation. One possible reason that could account for this phenomenon might be the presence of chelating compounds in R2A agar that sequester monochloramine present in tap water. Specifically, by chelating monochloramine, the residual disinfectant's ability in effecting toxicity on microbes in tap water could be drastically reduced. In particular, the new monochloramine-chelating compound complex would have reduced capability at inhibiting microbial viability in tap water. This could result in enhanced viability of microbes when tap water was inoculated on R2A agar. Lack of similar chelating compounds in the formulated colourless agar meant that monochloramine remains an active agent in the agar and inhibited viability of microbes; thereby, preventing their growth.

Viewed from a different perspective, microbes in deionized water originated from tap water feed, but adsorption of microbes to various surfaces of the deionized water production system such as the filter membrane help concentrate the microorganisms present in deionized water. Specifically, different microbes exhibited different growth and death cycles in tap and deionized water. With a filter membrane as surfaces to adhere to, microbes were concentrated in the deionized water production system in both numbers and types of microbes. Hence, it was natural to recover more types of microorganisms from deionized water compared to the tap water feed, given that microbes of low abundance in tap water could adhere to the surfaces of filter membrane, multiply and grow and constitute part of a sprawling biofilm matrix teeming with microorganisms.

Agar plate **Close-up**



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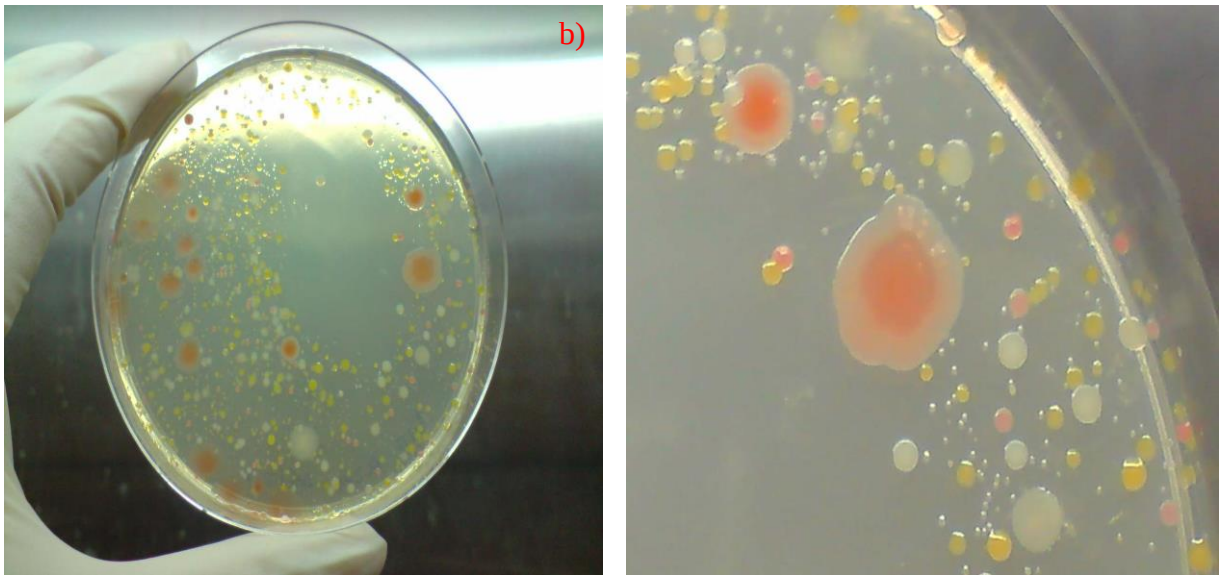

Figure 7: Time course profile of microbial growth on R2A agar from deionized water at 30 ^{o}C a) 1.5 days of incubation, and b) 8 days of incubation.

Due possibly to stress sustained from exposure to monochloramine in tap water, microbes in fresh deionized water often require a few days of incubation for growth. Shown in Figure 7 is a time course analysis of the progressive growth of microbes on R2A agar after multiple days of incubation. Specifically, relatively few number and types of colonies were observable on R2A agar after 1.5 days of incubation (Figure 7a). But, at 8 days of incubation, dense coverage of the agar plate with microbial colonies was observed (Figure 7b). Taken together, microbes in deionized water grew slowly and require multiple days of incubation for colony formation.

Further analysis of Figure 7b revealed that there was a clear zone in the middle of the agar plate devoid of microbial colonies. Such a clear zone was likely not due to the lack of inoculum in the area. Given the rectangular shape of the clear zone where multiple types of colonies were observed on the boundary of the zone, it was unlikely that toxic or inhibitory compounds were secreted by the microbes around the zone periphery since circular clear zones surrounding the colonies were not observed. What likely happened could be the lack of keystone species in the clear zone. Thus, there was no secretion of needed metabolites and signalling molecules into the agar medium, which could have enabled the growth of other microbes in the surrounding area.

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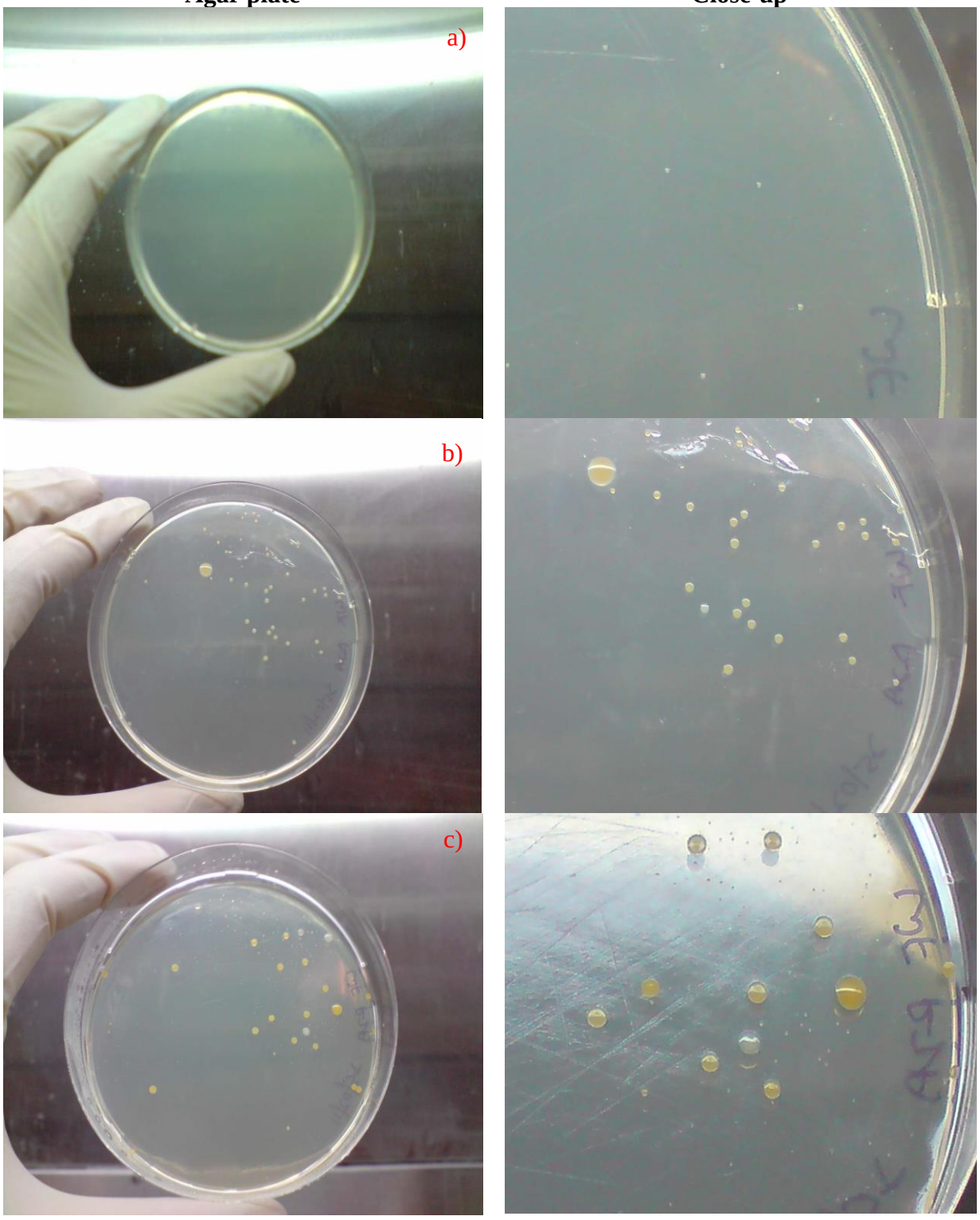
Agar plate **Close-up**



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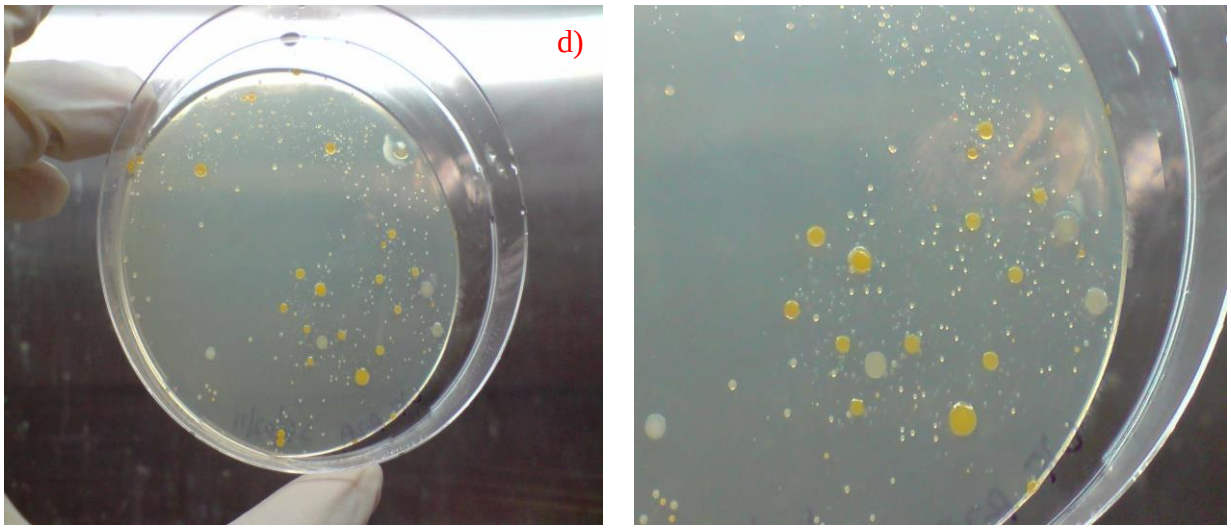
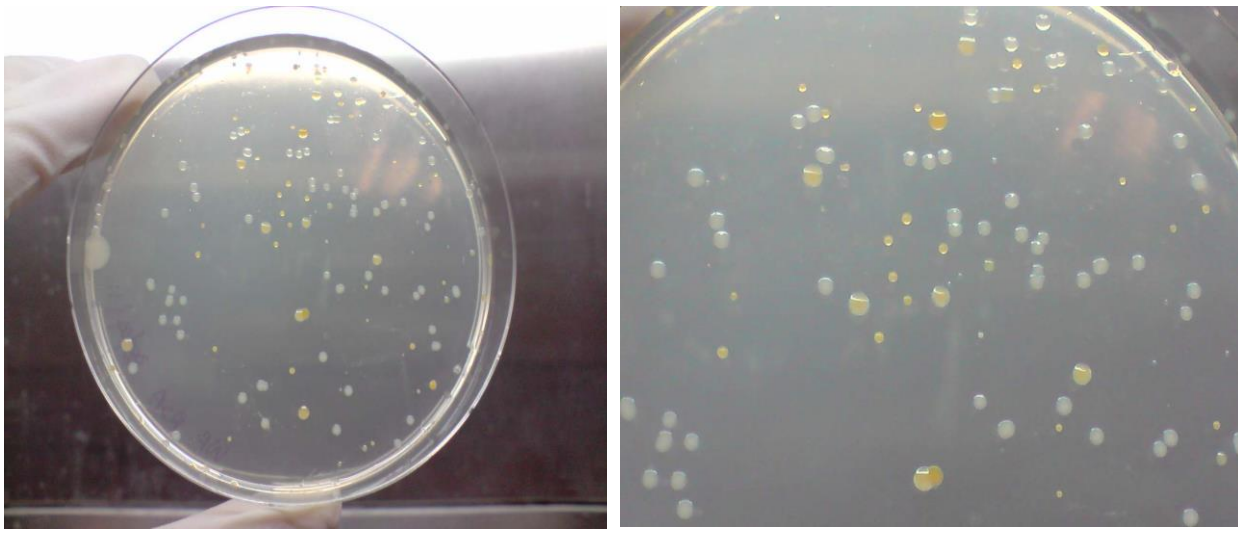


Figure 8: Growth of microbes from tap water on R2A agar at 30 ºC. a) 2 days of incubation, b) 3 days of incubation, c) 4 days of incubation, and d) 7 days of incubation.

Similar cultivation experiments with tap water as inoculum on R2A agar at 30 ºC incubation revealed that microbes in tap water were also slow-growing, which could likely be due to the effect of monochloramine's toxicity on the cells as well as a long adaptation period after microbes' regain of viability due to the sequestration of monochloramine by chelating compounds in R2A agar. Specifically, only small colonies were seen on R2A agar after 2 days of incubation (Figure 8a), which progressively increased in size with time (Figure 8b and 8c). More importantly, more types of colonies were recovered with increasing time of incubation. Finally, significant increase in types and numbers of colonies in close proximity to each other was observed after 7 days of incubation (Figure 8d); thereby, indicating that microbes exposed to monochloramine disinfectant in water required more time for cultivation and recovery on agar.



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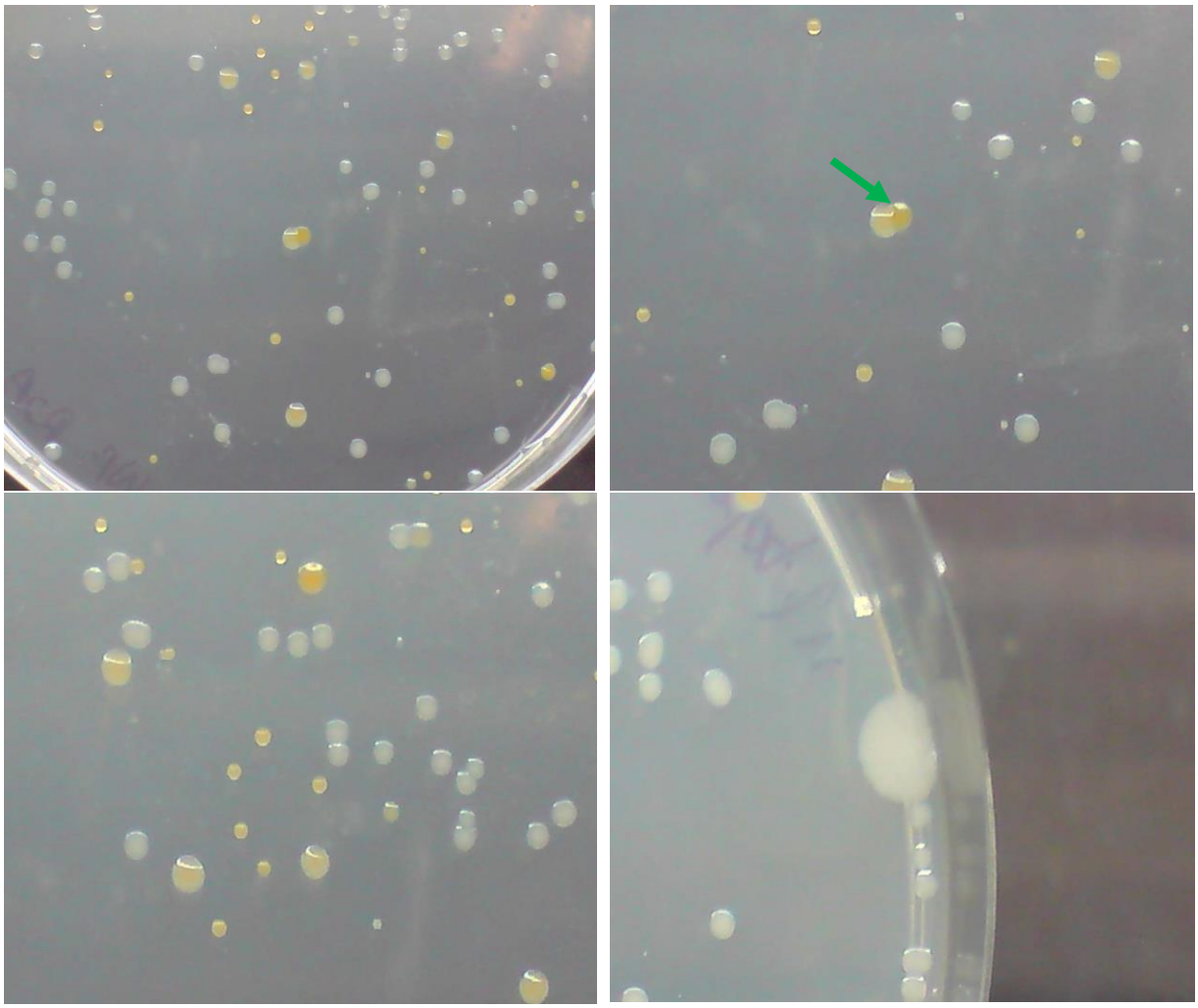

Figure 9: Growth of microorganisms from deionized water on R2A agar at 25 ºC after 4 days of incubation. Photos of agar plate and close-up pictures of the same plate.

Different types of microbes from deionized water were recovered on R2A agar after 4 days of incubation at 25 ºC (Figure 9). However, the types and numbers of microbes recovered were significantly fewer compared to multiple days of incubation on R2A agar at 30 ºC, which suggested that microbes in tap and deionized water could have adapted to tropical water temperature of around 30 ºC. Lack of clear zones around individual microbial colony as well as close proximity between different types of colonies suggested that growth inhibitory compounds were not secreted. More importantly, different types of colonies could be seen in contact with each other, which indicated that possible synergistic interactions or at least neutral relationships might exist between them (green arrow in Figure 9). Overall, microbes in deionized water that could be recovered on R2A agar at 25 ºC developed small round colonies not different from those exhibited by microbes recovered at 30 ºC on the same agar. Relative close proximity between colonies on R2A agar after 4 days of incubation at 25 ºC suggested that the consortium of microbes in deionized water did not exhibited significant antagonistic relationships, which suggested possibe selection forces in

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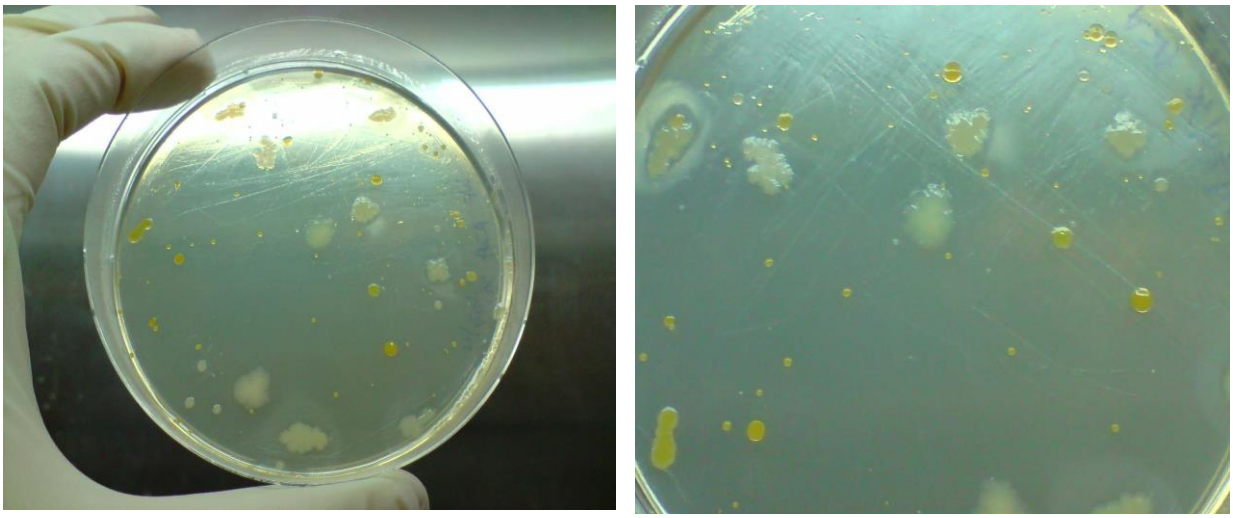
deionized water environment that selected for neutral, synergistic or mutualistic relationships in place of antagnoistic ones.

Figure 10: Relatively few microbial colonies were recovered from tap water on R2A agar at 25 ^{o}C after 4 days of incubation. Photo of agar plate and close-up pictures of the same plate.

Similar growth experiments for profiling microbes in tap water on R2A agar at 25 ^{o}C revealed few colonies (Figure 10); thereby, highlighting that microbes in tap water could have adapted to 30 ^{o}C temperature in distribution pipelines in a tropical climate. This suggested that natural selection could exert its effects, in this case, modulating the preferred growth temperatures of microbes in tap water, over a relatively short time span.



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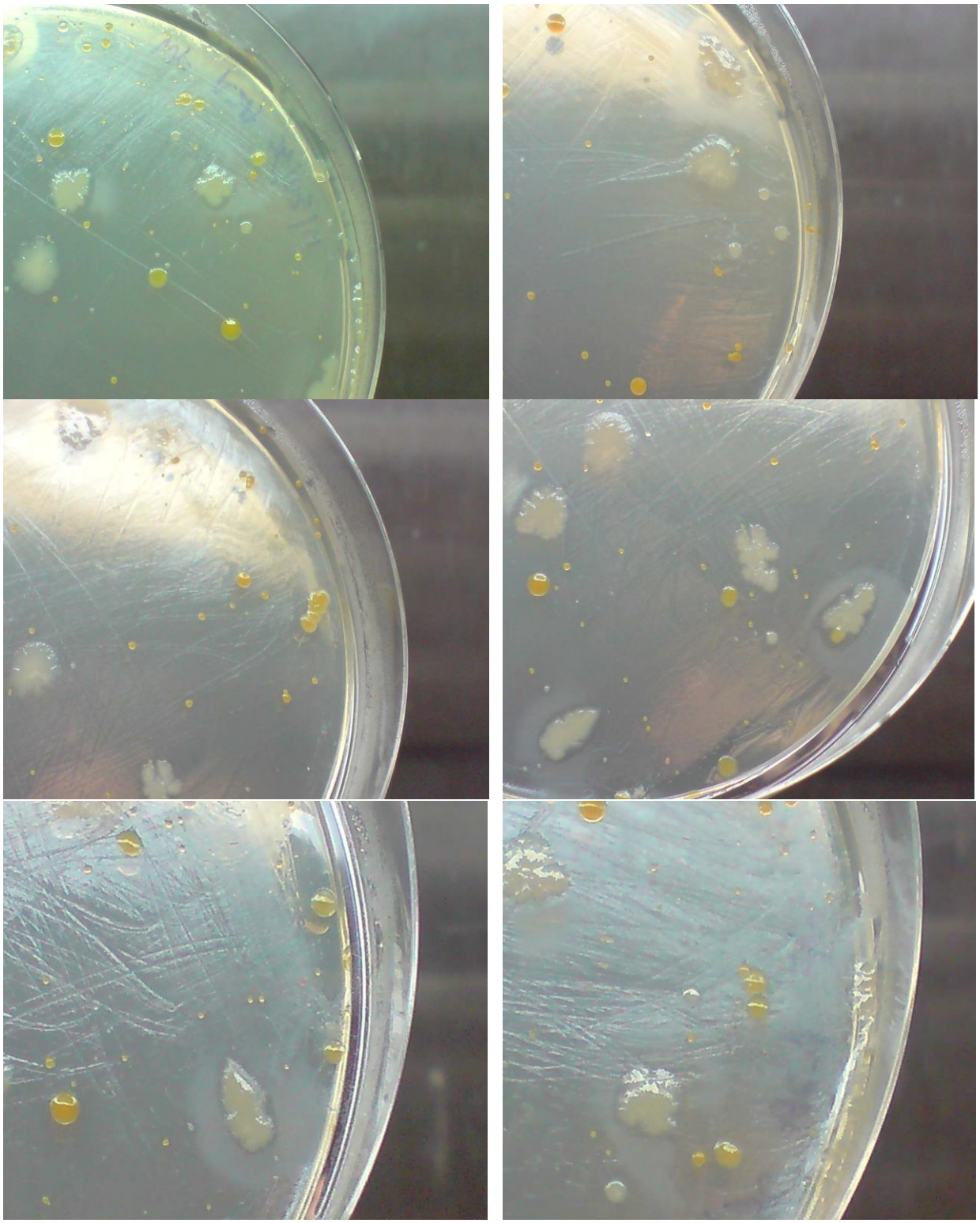

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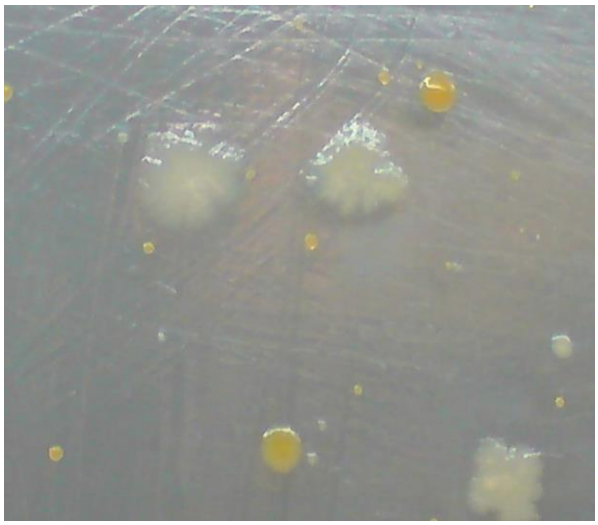

Figure 11: Growth of microbes in deionized water on R2A agar at 37 ^{o}C after 6 days of incubation. Photos of agar plate and close-up pictures of the same plate.

Multiple types of microbial colonies including fungus were recovered on R2A agar from deionized water after 6 days of incubation at 37 ^{o}C (Figure 11). Thus, these microbes could potentially colonize human host and were potential pathogens. Close examination of the agar plate revealed that the irregularly shaped white colony of a fungus likely secreted compounds into the surrounding medium resulting in a white halo around the colony. Yellow colonies were observed in close proximity to the fungal colonies; thereby, suggesting that secreted compounds did not affect the growth of the yellow colonies. Given that many species of fungus secrete antibiotics for self-defense, observed "immunity" of yellow colonies to secreted compounds of the white irregularly shaped fungal colonies raised the possibility of presence of antibiotic resistant microbes in the deionized water biofilm within the deionized water production system. In general, close proximity of many types of colonies to the fungal colonies revealed that resistance to the compounds secreted by the white fungal colonies could be prevalent in species of the microbial biofilm in deionized water production system. Additionally, fewer microbial colonies were recovered from deionized water on R2A agar at 37 ^{o}C incubation compared to 30 ^{o}C, which suggested that microbes in deionized and tap water might have adapted to tropical water temperatures of ~30 ^{o}C.

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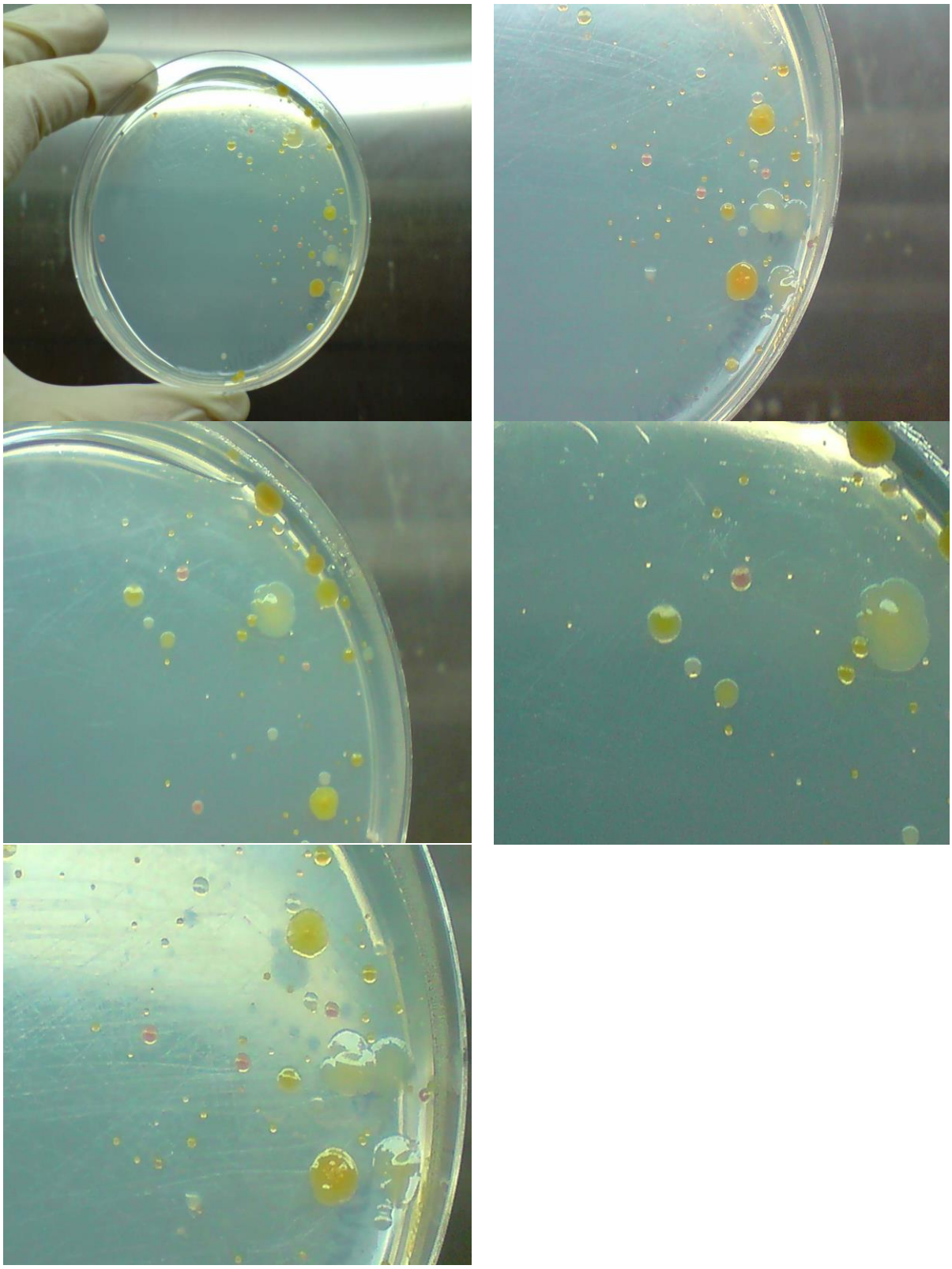

Figure 12: Growth of microbes in deionized water on formulated colourless agar with 1 g/L yeast extract after 6 days of incubation at 37 ºC. Photos of agar plate and close-up pictures of the same plate.

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Formulated colourless agar medium with 1 g/L of yeast extract also recovered multiple types of microbial colonies (including white irregularly shaped fungal colonies) after 6 days of incubation of deionized water at 37 ^{o}C (Figure 12). This reinforced the notion that potential human pathogens could be present in deionized water. In general, fewer types and numbers of colonies were recovered from deionized water during cultivation at 37 ^{o}C than at 30 ^{o}C due possibly to acclimation of microbes to tropical ambient temperatures of 30 ^{o}C. Colonies recovered on the colourless agar were in close proximity to each other; thereby, indicating that metabolites and signalling compounds secreted enabled the growth of other species of microbes. Many types of colonies were in close proximity to the white irregularly shaped fungal colonies, which did not have a white halo surrounding the colonies. This suggested that the fungal colonies were likely not antagonistic to other species.

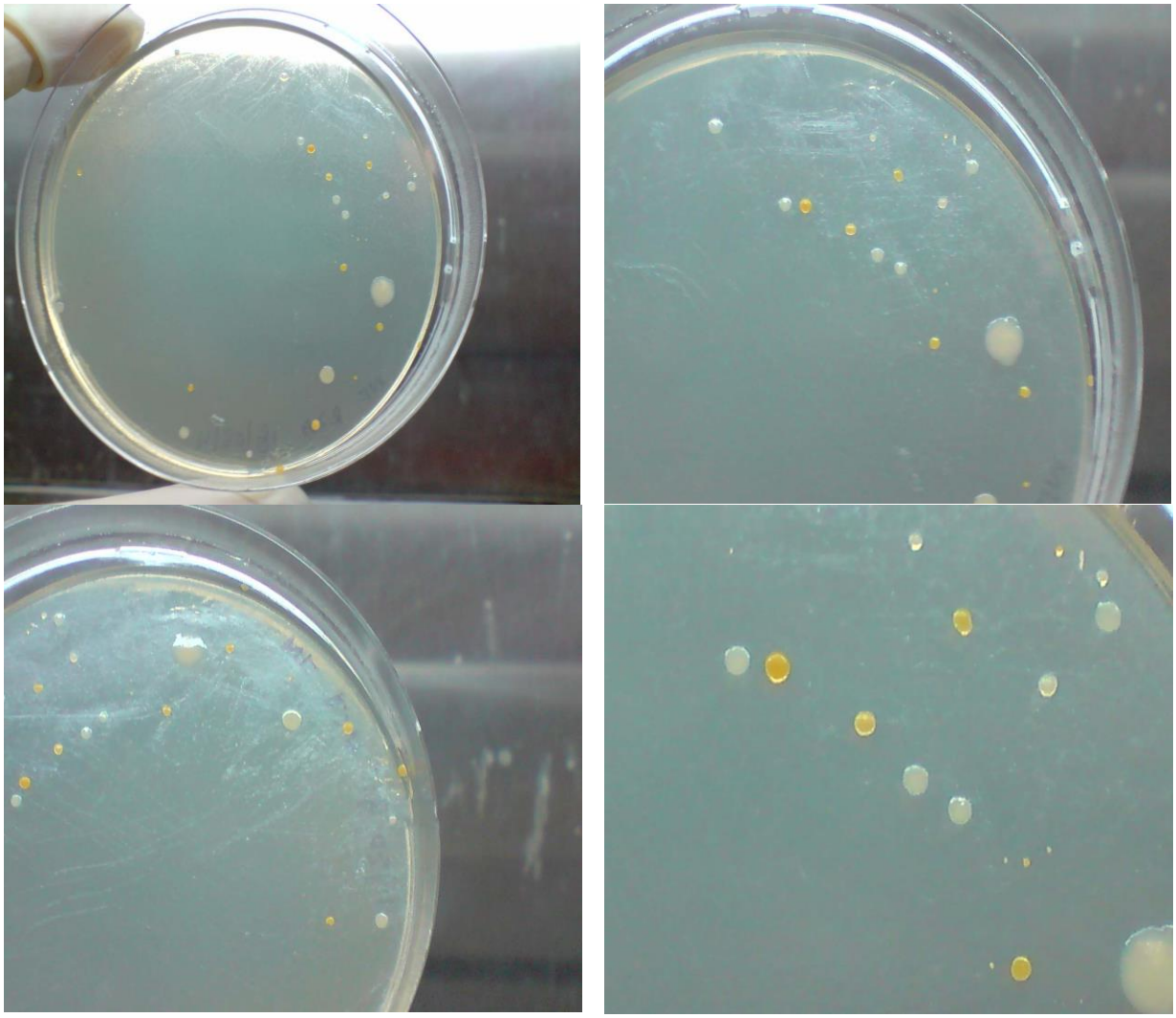

Figure 13: Growth of microorganisms in tap water on R2A agar after 7 days of incubation at 37 ^{o}C. Photo of agar plate with close-up pictures of the same plate.

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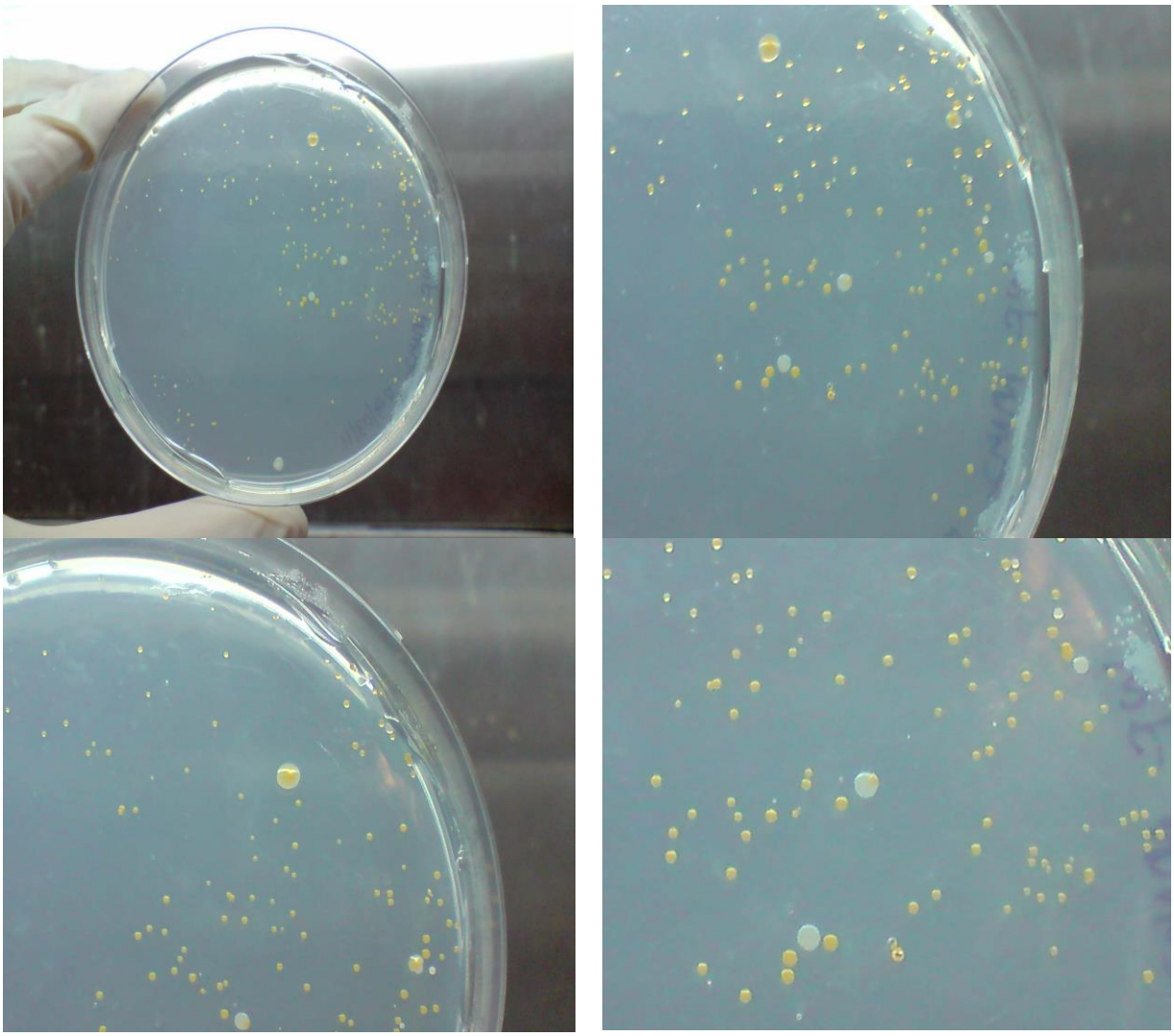
Similarly, microbial colonies were also recovered from tap water on R2A agar after 7 days of incubation at 37 ºC (Figure 13), which indicated that potential human pathogens were present in tap water. In general, fewer colonies were recovered at 37 ºC compared to 30 ºC on R2A agar, which provided further evidence that 30 ºC might be the preferred temperature of growth for microbes in tap water, similar to the case for microbes in deionized water. Recovered microbes were in close proximity to each other, with a large clear zone of the agar plate devoid of any colony, which suggested that the hypothesis of keystone microbial species secreting metabolite or signalling molecules that enabled the growth of neighbouring colonies in the agar matrix might be valid. Specifically, different types of colonies were seen in close proximity to each other on the agar, which suggested that inhibitory compounds were not present in the agar surrounding the colonies. Additionally, microbial colonies in close proximity to each other might require secreted metabolites or signalling molecules from other microbial species for growth. In general, close proximity of colonies on agar plate meant that neutral and synergistic relationships might be more prevalent compared to antagonistic ones.



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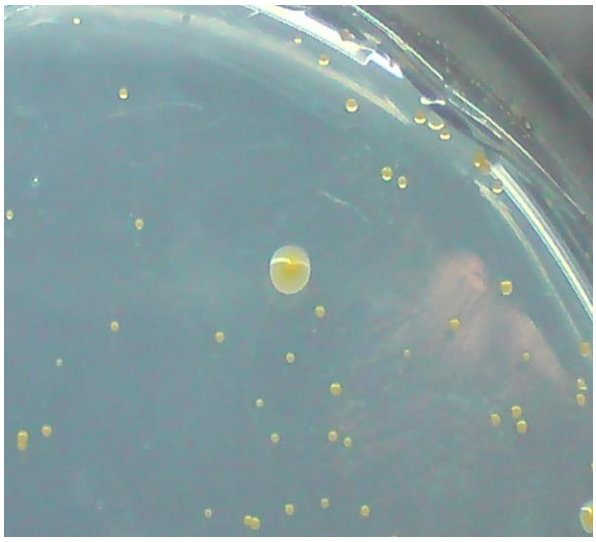

Figure 14: Growth of microorganisms from tap water on formulated colourless agar with 1 g/L yeast extract after 7 days of incubation at 37 ºC. Photo of agar plate with close-up pictures of the same plate.

Microbial colonies recovered from tap water on formulated colourless agar with 1 g/L of yeast extract after 7 days of incubation at 37 ºC (Figure 14) exhibited similar characteristics to those recovered on R2A agar under identical incubation conditions (Figure 13). Number and types of colonies recovered was significantly fewer at 37 ºC incubation compared to 30 ºC. Additionally, multiple types of microbial colonies were recovered on the formulated colourless agar medium in close proximity to each other, which suggested that antagonistic relationships were generally less important compared to neutral, synergistic and mutualistic relationships in the drinking water biofilm present in tap water distribution network. Additionally, inhibitory compounds were likely not secreted by the microbes recovered and there could be exchange of metabolites and signalling molecules between colonies.

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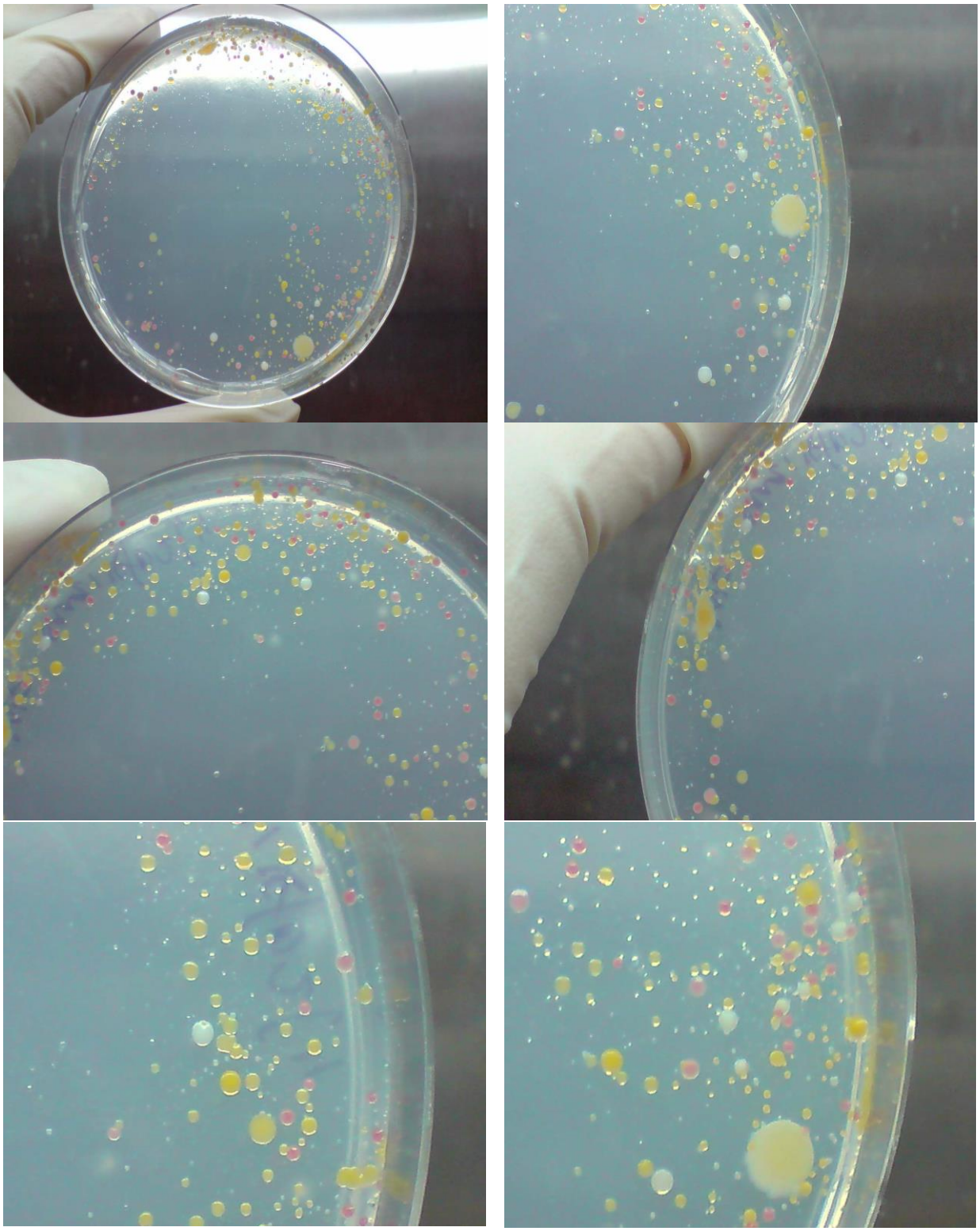

Figure 15: Growth of microorganisms from deionized water on formulated colourless agar without yeast extract after 8 days of incubation at 30 ^{o}C. Photo of agar plate with close-up pictures of the same plate.

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Given that ability to grow in minimal salts medium represents a significant growth advantage over other microbial species, cultivation of microbes from deionized water on formulated colourless agar without yeast extract was conducted for multiple days at 30 ^{o}C to understand the diversity of microbes able to grow without supplementation of vitamins and growth factors. Results indicated a rich diversity of different types of microbial species at differing relative abundance could grow on minimal salt version of formulated colourless agar (Figure 15); thereby, suggesting that a large consortium of microorganisms could collectively grow without infusion of vitamins and growth factors from the environment. Specifically, microbes in the community could share vitamins and growth factors secreted by some members of the consortium; thereby, gaining the ability to grow on a minimal salts medium. Hence, microbial species able to synthesize vitamins and growth factors without uptake from the environment play pivotal roles in enabling the viability of the microbial consortium in a minimal salts medium. For example, large clear zones devoid of microbial colonies in the centre of the agar plate suggested that microbial species able to grow in minimal salts medium and which secrete vitamins and growth factors into the surrounding medium were absent in the zone.

Conclusions

Multiple types of microbial colonies at high cell density was recovered from deionized and tap water on R2A agar and a formulated colourless agar during multi-day incubation at 25, 30 and 37 ^{o}C. Greater diversity of microbial species of higher relative abundance were recovered from both deionized and tap water during growth at 30 ^{o}C compared to 25 and 37 ^{o}C; thereby, suggesting that microbes in deionized and tap water could have adapted to ambient tropical temperature of ~30 ^{o}C. This further indicated the relative speed at which natural selection could have exerted its effect in influencing the optimal growth temperatures of microbes in environmental matrixes such as freshwater. Recovery of microbes from deionized and tap water during incubation at 37 ^{o}C suggested that potential human pathogens could be in deionized and tap water.

More importantly, more types and higher colony density of microbes could be recovered on both agars from deionized water compared to tap water, due possibly to the removal of the residual disinfectant, monochloramine, from tap water through adsorption on ion exchange resins of the deionized water production system. Removal of monochloramine thus reduced a significant environmental stressor that impinge on microbial viability. Additionally, during cultivation of microbes from tap water, more colonies of different types could be recovered on R2A agar compared to the formulated colourless agar, due probably to the presence of chelating compounds in R2A agar able to sequester monochloramine; thereby, reducing its toxicity to cells. Similar chelating compounds were unlikely to be present in the formulated colourless agar medium.

Rich diversity of microbes was recovered at high colony density from deionized water on both R2A and formulated colourless agar, which could be due to the role of the filter membrane

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of the deionized water production system serving as surfaces for the adsorption and concentration of microbes. Thus, biofilms likely formed on these surfaces and constituted a community of microbes that could harbour microbial species previously not viable in deionized or tap water. Such microbes could regain viability through the metabolites or signalling factors secreted by other microbial species. More importantly, different microbial species of low relative abundance could also be concentrated in such biofilms, which help explained the large diversity of microbes recovered from deionized water.

Close proximity of microbial colonies on R2A agar and formulated colourless agar at high colony density revealed that antagonistic relationships were likely less prevalent between species compared to neutral or cooperative relationships. For example, metabolites or signalling molecules secreted by a keystone species could enable the return to viability of microbes which previously chose a "hibernation" cellular differentiation programme due to presence of monochloramine in tap water. Thus, a consortium of microbes could be recovered in close proximity to the keystone species through an exchange of metabolites or signalling molecules, resulting in areas of the agar plate with dense microbial colonies and other areas devoid of microbes where the keystone species was absent. In essence, need for survival in nutrient-poor deionized water likely selected for cooperative or neutral relationships between microbes in the biofilm present in deionized water production system and drinking water distribution pipelines, which explained the relatively lack of antagonistic relationships between microbial species recovered.

Finally, cultivation on agar plates through the spread plate technique represent a simple and effective method for assessing the microbiological quality of tap and deionized water, and which together with conductivity and resistivity measurements, comprise a trio of tests useful for maintaining the quality of deionized water.

Supplementary information

Photos of agar plates in other experiments could be found in the appended supplementary information.

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Conflicts of interest

The author declares no conflicts of interest.

Funding

The author thank the National University of Singapore for financial support.

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