applied biosciences

MDPI

Communication

Sedimentation Rate of *Dunaliella salina* in Dark Conditions

Angelica Naka * and Midori Kurahashi

Graduate School of Agricultural and Life Sciences, The University of Tokyo, Tokyo 113-0032, Japan
* Correspondence: angelica.naka@mail.ecc.u-tokyo.ac.jp

Abstract: Microalgae are a source of carbohydrates, proteins and lipids. Thus, they can be considered as raw material to transition from current fossil fuel-based refineries to biorefineries. Microalgae harvesting is considered a major challenge in biomass production. There are several harvesting techniques, but the majority of them are either expensive or not effective. The harvesting method that we propose is sedimentation-induced by light blockage, taking advantage of the motility characteristics of certain microalgae. In this research, the halophilic microalgae *Dunaliella salina* was selected. Experiments were conducted under light and dark conditions to compare the sedimentation rates. Sedimentation behavior was measured by collecting data on the optical density and cell count under both light and dark conditions. The results showed that, under light conditions, the cell count in the middle of the flask decreased from 1×10^6 cell/mL to 5×10^4 cell/mL after 50 days. Under dark conditions sedimentation took less than 10 days for complete settlement. Leaving *Dunaliella salina* under dark conditions may constitute a promising harvest method as this provides a high recovery rate and requires low energy.

Keywords: *Dunaliella salina*; microalgae harvesting; gravity sedimentation

Citation: Naka, A.; Kurahashi, M. Sedimentation Rate of *Dunaliella salina* in Dark Conditions. *Appl. Biosci.* **2023**, *2*, 14–20. https://doi.org/10.3390/applbiosci2010002

Academic Editor: Robert Henry

Received: 13 November 2022
Revised: 21 December 2022
Accepted: 4 January 2023
Published: 10 January 2023

1. Introduction

Microalgae are photosynthetic microorganisms that can be found in marine or freshwater environments. They convert sunlight, water, nutrients and carbon dioxide to produce microalgal biomass that consists of carbohydrates, proteins, lipids and other compounds over short periods of time. Microalgal biomass can be considered a renewable and sustainable source of raw material for different industries such as food, feed, chemical, fermentation, pharmaceutical, etc. [1–5]. They do not compete with agriculture for arable land as they can be cultivated in areas that are arid or affected by drought or salinity [6–8]. Some of them grow in saline water or wastewater, which do not represent any threat to freshwater supply [9]. They also have a short period of harvesting (1 to 10 days), and can be harvested practically year round [6]. Therefore, microalgae can help make the transition from current fossil fuel-based refineries to biorefineries in order to tackle climate change by creating a carbon neutral society. To reduce CO_2 emissions from the current fossil fuel use and reach carbon neutrality, massive amounts of microalgae become necessary.

Except for large species such as *Arthrospira*, microalgae are too small (2 to 20 µm) for conventional filtration systems. Therefore, a major challenge in biomass production lies in their harvesting [10–17], mainly because of the high cost and energy demand [18]. For instance, a research team reported that harvesting was 20–30% of the total cost of biomass production [19]. Microalgae harvesting results in 50 to 200 fold concentration of algae [20] and can be performed by mechanical, chemical, biological or electrical methods [21,22]. Some of the harvesting methods include gravity sedimentation, flocculation, flotation, filtration and screening, centrifugation, electricity-based techniques, ultrasound and immobilization, including combinations [17,22–26]. The majority of them are expensive and not very efficient, requiring long extraction periods or the use of chemicals [23,27]. Thus, there is a need to seek eco-friendly, cost-efficient, and low-energy systems.

Sedimentation appears to be one of the pre-dewatering biomass preconcentration methods with the lowest cost and energy consumption [11,28]. The settlement can be described by Stokes' Law (Equation (1)) which describes the velocity of a sphere falling in a fluid in terms of the radius of the cells and the difference in density between the microalgae cells and the medium:

$$v = \frac{2}{9} g \frac{r^2}{\eta}(\rho_s - \rho_l) \quad (1)$$

where v is the settlement velocity, g is the acceleration due to gravity (9.81 m/s^2), r is the spherical particle radius, η is the dynamic viscosity and ρ_s and ρ_l are the solid (particle) and liquid (medium) densities, respectively.

In this research, the halophilic microalgae *Dunaliella salina* was selected because of the high salinity that is required for its growth (up to 5 M NaCl), making it suitable for open culture systems while reducing the risk of contamination from other microalgae and/or protozoa. Outdoor systems reduce the production cost because of freely available sunlight, while being suitable only for microalgae that can live in extreme conditions such as high acidity, alkalinity or salinity. *Dunaliella salina* is well known as a source of beta carotene that is produced when exposed to harsh conditions especially in terms of light, salinity, temperature and nutrients. It also produces a wide range of compounds with different potential applications. We propose the massive and open culture of this microalgae on non-arable land to use as raw materials in different industries and be able to make the transition to a biorefinery. Considering that *Dunaliella salina* is a motile microalgae, it can probably be assumed that light blockage may affect its motility which may lead to settlement. There is research on the effect of carbon dioxide and pH on motility of flagellated microalgae, including *Dunalliela salina* [29]. However, to the best knowledge of the authors, there is no research that studies sedimentation speed and compares settlement rates in light and dark conditions. Therefore, this research was conducted to monitor *Dunaliella salina* sedimentation with time, using optical density sensors in both light and dark conditions.

2. Materials and Methods

The microalgae *Dunaliella salina* (strain CS-744/01, from the Australian National Algae Culture Collection, isolated from a saline lake in Western Australia) was used in the settlement experiments. The microalgae were cultivated in f/2 medium [30,31] with a salinity of 12% (2 M NaCl). The f/2 medium was prepared by mixing all the chemicals in sea salt (Red Sea Salt used in aquariums) solution. A pre-culture of 400 mL was inoculated into 3600 mL of fresh medium in a 5 L beaker and incubated at 25 °C under 200 µmol/m^2/s of continuous illumination (white LED) for 10 days without agitation. The initial cell concentration was 5.0×10^4 cell/mL, and the pH was 7.14. The 5 L beaker was lightly covered with a plastic film to prevent evaporation. The pH was measured using a portable pH meter (Hanna Instruments HI98130), and the salinity was measured with a portable salt refractometer (Tekcoplus Refractometer ATC). The settlement experiments were conducted when the cell concentration was around 1×10^6 cell/mL. *Dunaliella salina* was placed in 25 cm^2 culture flasks with double sealed caps (canted neck, slim type, Iwaki). Flasks were filled to the top (approximately 73 mL) just before overflowing and then they were closed with the caps. The settlement of *Dunaliella salina* was tested in bright and dark conditions. To monitor the settlement speed of the microalgae, optical density (OD_{600}) equipment (Taitec OD-Monitor A&S connected to OD sensor-S) with light transmission (horizontal projection, 0.00 to 2.55) was used. The data were taken every minute and automatically stored on a USB attached to the system. The cell concentration was periodically monitored by taking a 25 µL sample from the middle of the flask and measured in the Invitrogen Tali Image Based Cytometer. Experiments were conducted until settlement was complete or OD values were close to zero. Thus, tests performed in dark conditions were monitored for 10 days, whereas experiments conducted in light conditions were monitored for 70 days. To obtain a consistent result, experiments were repeated four times for dark conditions and three times for light conditions.

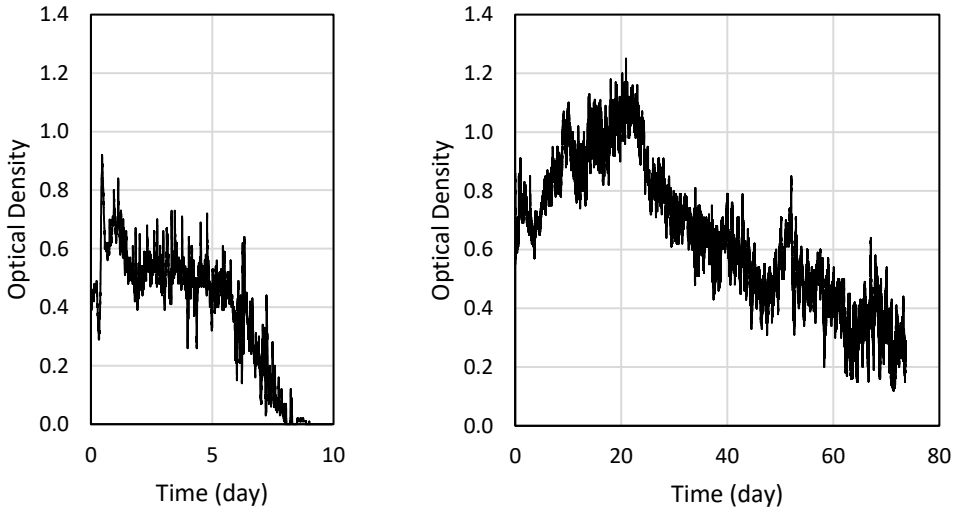
3. Results

The settlement experiments were conducted when the average concentration of *Dunaliella salina* was 1.28 × 10^6 cell/mL, the cell size was 12.5 µm and the pH was 8.03. The settlement or sinking of *Dunaliella salina* to the bottom of the flask took place gradually (Figure 1).

(**a**) (**b**) (**c**)

Figure 1. Picture of the settlement process under dark condition: (**a**) initial condition, (**b**) during settlement (on the 6th day), and (**c**) after settlement (on the 10th day).

Optical density (OD_{600}) change under dark and light conditions are presented in Figure 2. Optical density is generally used to measure culture growth, but in this study, we used it to determine the microalgae sinking rate. When *Dunaliella salina* was in suspension, the OD was between 0.2 and 1. However, when the microalgae settled, the OD was below 0.2. The OD change graphs that we obtained were very similar, and thus, only one result for each case was presented. Experiments conducted under dark conditions showed that *Dunaliella salina* settlement was completed after 8 days, while experiments conducted in light conditions showed that sinking took around 50 days.



(**a**) (**b**)

Figure 2. Optical density (OD) changes in (**a**) dark and (**b**) light conditions with time.

In order to study the cell concentration change with time, total cell concentration was measured periodically by taking 25 µL samples from the middle of the flask for both dark and light conditions. The results are presented in Figure 3. The results showed that under dark conditions, the microalgae concentration decreased to 2×10^4 cell/mL after 8 days, which correlates with biomass settlement. On the other hand, for light conditions, after 50 days, the cell concentration decreased to 5×10^4 cell/mL. Considering that the initial cell concentration of microalgae was 1.28×10^6 cell/mL, for dark conditions, it decreased by 98% after 8 days, and with light conditions, it took 50 days to reach 96%. When comparing both results, it can be said that gravity sedimentation of *Dunaliella salina* occurs faster in darker conditions rather than light available conditions. This is probably because blocking light decreases microalgae movement, making it sink. Gravity sedimentation is a simple harvesting method, but if carried out under dark conditions, it also reduces time from an accelerated sinking rate. Therefore, in terms of time and cost reduction, it is recommended to harvest *Dunaliella salina* under dark conditions.

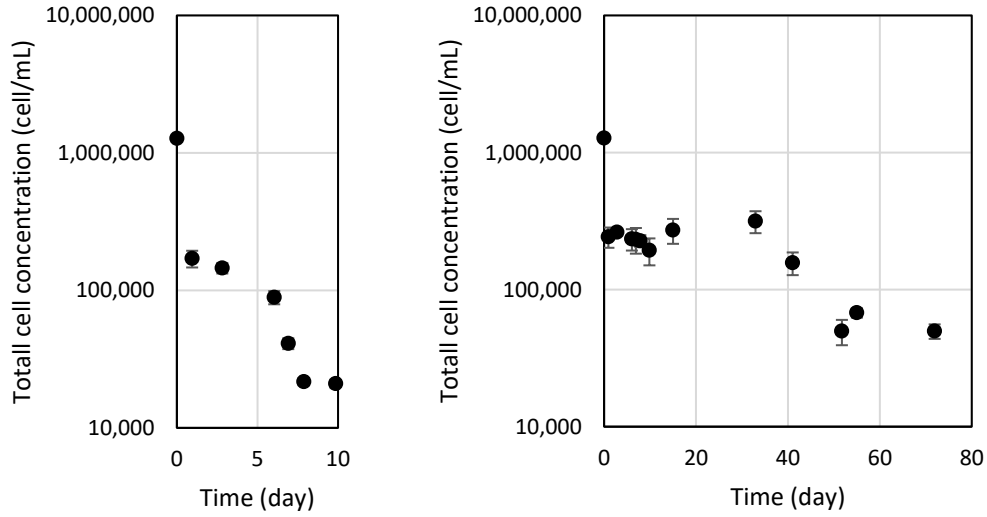


Figure 3. *Dunaliella salina* total cell concentration in (**a**) dark and (**b**) light conditions with time.

Stokes' law (Equation (1)) was used to predict the settlement velocity of *Dunaliella salina*. The density and the viscosity of the medium, obtained from the literature for seawater with 12% salinity, were considered to be 1090.25 kg/m^3 and 1320.6 µPa·s, respectively [32]. According to previous research, the density of the cytoplasm of marine microalgae ranges between 1030 and 1100 kg/m^3 [33]. Another study considered the density of *Dunaliella salina* as 1260 kg/m^3, the density of glycerol [34]. Due to the small density difference between the microalgae and the medium, it can be said that density contribution to settlement is small compared to the size of the microalgae. Stoke's law holds for spherical shaped microalgae, so *Dunaliella salina* was assumed to be spherical. The average size of *Dunaliella salina* was 12.5 µm, so for a radius of 6.25 µm, the settlement velocity is expected to be around 0.05 m/day. This value is not in accordance with experimental results probably because *Dunaliella salina* is a motile microalgae, which makes it more difficult to predict its settlement. Flagella are considered a dynamic morphological means to suspension [33], and thus, complex hydromechanical [35] and biophysical [36] parameters might be considered for settlement prediction models.

4. Discussion

Microalgae can potentially be a great source of raw materials such as protein, carbohydrates and lipids. However, its elevated price compared with raw materials from fossil fuel is still a great barrier to overcome. Therefore, reducing biomass production and processing costs are crucial for large scale culture and establishing a microalgae-based biorefinery. For instance, cost effective cultivation and harvesting methods may drastically reduce their cost. In this study, we proposed the gravity sedimentation technique of the halophilic microalgae *Dunaliella salina* under dark conditions taking advantage of its motility characteristic. In our previous study, we presented the settlement degree of *Dunaliella salina* at different temperatures and found a sedimentation rate of 79 to 96% between 20 to 30 degrees Celsius [37]. Thus, in the present study, we conducted experiments at 25 degrees Celsius and monitored the speed of settlement when experiments were conducted both in dark and light conditions, using OD and cell concentration data. Motile phytoplankton sank to the bottom of the flask when they lost their flagella, when stressed physiologically or with increasing age (senescent cells) [33]. In this study, light blockage might have triggered *Dunaliella salina*'s loss of movement and subsequent gravity settlement, but further research should be conducted to determine the mechanism involved. Light, photoperiod and nutrients may affect suspension and sinking, but these factors have not been well studied, so their level of influence are, to a certain degree, unknown [33]. Another hypothesis that can potentially explain the sinking of this microalgae is a possible aggregation between cells [33], especially when exposed to stressful conditions. However, further research should be conducted to test this hypothesis.

Costs can be kept low as the energy required for this harvesting method is low, the recovery rate is high, and no consumables are required. As such, it can probably constitute a cost-effective recovery method. In addition, chemical substances such as flocculants and pH adjusters are not necessary, which avoid chemical contamination. Physical and mechanical methods such as centrifugation, filtration (filter), evaporation to dryness, voltage application, etc. are not required, and equipment operation or maintenance are not necessary. This method is simple, suppressing costs.

For high volumes of microalgae or open and large-scale cultivation, we suggest temporarily transferring the culture to a collection container, then blocking the light for several days. The culture water can be removed by decantation or other methods, and the supernatant can be reused. This research was conducted in a laboratory under controlled conditions. As part of further research, we need to conduct experiments at a larger scale, performing the experiments outdoors to assure the efficacy of this method under a broad range of temperatures.

5. Conclusions

Sedimentation under dark conditions of *Dunaliella salina* was completed in less than 10 days, whereas settlement under light conditions took more than 50 days. The cell count in the middle of the flask decreased from 1×10^6 cell/mL to 5×10^4 cell/mL after 50 days (96% recovery rate). Under dark conditions, sedimentation took less than 10 days for almost complete settlement (98% recovery rate). Leaving *Dunaliella salina* under dark conditions may constitute a promising harvest method as this provides a high recovery rate, requires low energy and has high efficiency. A large-scale pilot is necessary before its application.

Author Contributions: Conceptualization, A.N. and M.K.; methodology, A.N. and M.K.; validation, A.N. and M.K.; formal analysis, A.N. and M.K.; investigation, A.N. and M.K.; resources, M.K.; data curation, A.N. and M.K.; writing—original draft preparation, A.N. and M.K.; writing—review and editing, A.N. and M.K.; visualization, A.N. and M.K.; supervision, M.K.; project administration, M.K. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data is contained within the article.

Acknowledgments: We want to express our deepest appreciation to Mitsubishi Gas Chemical Company for donating the Taitec OD Monitor A&S and OD sensor-S that were used for all experiments.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Chisti, Y. Society and microalgae: Understanding the past and present. In *Microalgae in Health and Disease Prevention*; Elsevier: Amsterdam, The Netherlands, 2018; pp. 11–21.
2. Zhuang, D.; He, N.; Khoo, K.S.; Ng, E.-P.; Chew, K.W.; Ling, T.C. Application progress of bioactive compounds in microalgae on pharmaceutical and cosmetics. *Chemosphere* **2021**, *291*, 132932. [CrossRef] [PubMed]
3. Yarkent, Ç.; Gürlek, C.; Oncel, S.S. Potential of microalgal compounds in trending natural cosmetics: A review. *Sustain. Chem. Pharm.* **2020**, *17*, 100304. [CrossRef]
4. Harun, R.; Danquah, M.K.; Forde, G.M. Microalgal biomass as a fermentation feedstock for bioethanol production. *J. Chem. Technol. Biotechnol.* **2010**, *85*, 199–203. [CrossRef]
5. Nagarajan, D.; Varjani, S.; Lee, D.-J.; Chang, J.-S. Sustainable aquaculture and animal feed from microalgae–Nutritive value and techno-functional components. *Renew. Sustain. Energy Rev.* **2021**, *150*, 111549. [CrossRef]
6. Schenk, P.M.; Thomas-Hall, S.R.; Stephens, E.; Marx, U.C.; Mussgnug, J.H.; Posten, C.; Kruse, O.; Hankamer, B. Second generation biofuels: High-efficiency microalgae for biodiesel production. *Bioenergy Res.* **2008**, *1*, 20–43. [CrossRef]
7. Vanthoor-Koopmans, M.; Wijffels, R.H.; Barbosa, M.J.; Eppink, M.H. Biorefinery of microalgae for food and fuel. *Bioresour. Technol.* **2013**, *135*, 142–149. [CrossRef]
8. Chew, K.W.; Yap, J.Y.; Show, P.L.; Suan, N.H.; Juan, J.C.; Ling, T.C.; Lee, D.-J.; Chang, J.-S. Microalgae biorefinery: High value products perspectives. *Bioresour. Technol.* **2017**, *229*, 53–62. [CrossRef] [PubMed]
9. Pessôa, L.C.; Deamici, K.M.; Pontes, L.A.M.; Druzian, J.I.; de Jesus Assis, D. Technological prospection of microalgae-based biorefinery approach for effluent treatment. *Algal Res.* **2021**, *60*, 102504. [CrossRef]
10. Schlesinger, A.; Eisenstadt, D.; Bar-Gil, A.; Carmely, H.; Einbinder, S.; Gressel, J. Inexpensive non-toxic flocculation of microalgae contradicts theories; overcoming a major hurdle to bulk algal production. *Biotechnol. Adv.* **2012**, *30*, 1023–1030. [CrossRef]
11. Vandamme, D.; Foubert, I.; Muylaert, K. Flocculation as a low-cost method for harvesting microalgae for bulk biomass production. *Trends Biotechnol.* **2013**, *31*, 233–239. [CrossRef]
12. Talukder, M.M.R.; Das, P.; Wu, J.C. Immobilization of microalgae on exogenous fungal mycelium: A promising separation method to harvest both marine and freshwater microalgae. *Biochem. Eng. J.* **2014**, *91*, 53–57. [CrossRef]
13. Estime, B.; Ren, D.; Sureshkumar, R. Cultivation and energy efficient harvesting of microalgae using thermoreversible sol-gel transition. *Sci. Rep.* **2017**, *7*, 1–9. [CrossRef]
14. Poelman, E.; De Pauw, N.; Jeurissen, B. Potential of electrolytic flocculation for recovery of micro-algae. *Resour. Conserv. Recycl.* **1997**, *19*, 1–10. [CrossRef]
15. Chen, C.-Y.; Yeh, K.-L.; Aisyah, R.; Lee, D.-J.; Chang, J.-S. Cultivation, photobioreactor design and harvesting of microalgae for biodiesel production: A critical review. *Bioresour. Technol.* **2011**, *102*, 71–81. [CrossRef]
16. Najjar, Y.S.H.; .Abu-Shamleh, A. Harvesting of microalgae by centrifugation for biodiesel production: A review. *Algal Res.* **2020**, *51*, 102046. [CrossRef]
17. Li, S.; Hu, T.; Xu, Y.; Wang, J.; Chu, R.; Yin, Z.; Mo, F.; Zhu, L. A review on flocculation as an efficient method to harvest energy microalgae: Mechanisms, performances, influencing factors and perspectives. *Renew. Sustain. Energy Rev.* **2020**, *131*, 110005. [CrossRef]
18. Brennan, L.; Owende, P. Biofuels from microalgae—A review of technologies for production, processing, and extractions of biofuels and co-products. *Renew. Sustain. Energy Rev.* **2010**, *14*, 557–577. [CrossRef]
19. Gudin, C.; Thepenier, C. Bioconversion of solar energy into organic chemicals by microalgae. *Adv. Biotechnol. Process. (USA)* **1986**, *6*, 73–110.
20. Molina Grima, E.; Belarbi, E.-H.; Acién Fernández, F.G.; Robles Medina, A.; Chisti, Y. Recovery of microalgal biomass and metabolites: Process options and economics. *Biotechnol. Adv.* **2003**, *20*, 491–515. [CrossRef]
21. Morais Junior, W.G.; Gorgich, M.; Corrêa, P.S.; Martins, A.A.; Mata, T.M.; Caetano, N.S. Microalgae for biotechnological applications: Cultivation, harvesting and biomass processing. *Aquaculture* **2020**, *528*, 735562. [CrossRef]
22. Singh, G.; Patidar, S. Microalgae harvesting techniques: A review. *J. Environ. Manag.* **2018**, *217*, 499–508. [CrossRef]
23. Goh, B.H.H.; Ong, H.C.; Cheah, M.Y.; Chen, W.-H.; Yu, K.L.; Mahlia, T.M.I. Sustainability of direct biodiesel synthesis from microalgae biomass: A critical review. *Renew. Sustain. Energy Rev.* **2019**, *107*, 59–74. [CrossRef]
24. Ho, S.-H.; Chen, C.-Y.; Lee, D.-J.; Chang, J.-S. Perspectives on microalgal CO_2-emission mitigation systems—A review. *Biotechnol. Adv.* **2011**, *29*, 189–198. [CrossRef]
25. Kim, J.; Yoo, G.; Lee, H.; Lim, J.; Kim, K.; Kim, C.W.; Park, M.S.; Yang, J.-W. Methods of downstream processing for the production of biodiesel from microalgae. *Biotechnol. Adv.* **2013**, *31*, 862–876. [CrossRef]

26. Uduman, N.; Qi, Y.; Danquah, M.K.; Forde, G.M.; Hoadley, A. Dewatering of microalgal cultures: A major bottleneck to algae-based fuels. *J. Renew. Sustain. Energy* **2010**, *2*, 012701. [CrossRef]
27. Besson, A.; Guiraud, P. High-pH-induced flocculation–flotation of the hypersaline microalga *Dunaliella salina*. *Bioresour. Technol.* **2013**, *147*, 464–470. [CrossRef]
28. Milledge, J.J.; Heaven, S. A review of the harvesting of micro-algae for biofuel production. *Rev. Environ. Sci. Bio/Technol.* **2013**, *12*, 165–178. [CrossRef]
29. Wang, Y.; Fan, X.; Gao, G.; Beardall, J.; Inaba, K.; Hall-Spencer, J.M.; Xu, D.; Zhang, X.; Han, W.; McMinn, A. Decreased motility of flagellated microalgae long-term acclimated to CO_2-induced acidified waters. *Nat. Clim. Change* **2020**, *10*, 561–567. [CrossRef]
30. Guillard, R.R. Culture of phytoplankton for feeding marine invertebrates. In *Culture of Marine Invertebrate Animals*; Springer: Berlin/Heidelberg, Germany, 1975; pp. 29–60.
31. Guillard, R.R.; Ryther, J.H. Studies of marine planktonic diatoms: I. *Cyclotella nana* Hustedt, and Detonula confervacea (Cleve) Gran. *Can. J. Microbiol.* **1962**, *8*, 229–239. [CrossRef]
32. Leyendekkers, J. Prediction of the density and viscosity of seawater, its concentrates and other multicomponent solutions using the Tammann-Tait-Gibson (TTG) model. *Desalination* **1979**, *29*, 263–274. [CrossRef]
33. Smayda, T.J. The suspension and sinking of phytoplankton in the sea. *Oceanogr. Mar. Biol. Ann. Rev.* **1970**, *8*, 353–414.
34. Xu, Y.; Milledge, J.J.; Abubakar, A.; Swamy, R.; Bailey, D.; Harvey, P. Effects of centrifugal stress on cell disruption and glycerol leakage from *Dunaliella salina*. *Microalgae Biotechnol.* **2015**, *1*, 20–27. [CrossRef]
35. Ludwig, W. Zur theorie der flimmerbewegung (dynamik, nutzeffekt, energiebilanz). *Z. Für Vgl. Physiol.* **1930**, *13*, 397–504. [CrossRef]
36. Jahn, T.L.; Bovee, E.C. Motile behavior of protozoa. In *Research in Protozoology*; Elsevier: Amsterdam, The Netherlands, 1967; pp. 41–200.
37. Watsuji, T.-o.; Naka, A.; Morita, Y.; Kurahashi, M. Effect of temperature and dissolved oxygen on gravity sedimentation of the unicellular alga *Dunaliella salina*. *Ann. Microbiol.* **2021**, *71*, 1–7.