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Impact of Perfluorocarbons with Gas Transport Function on Growth of Phototrophic Microorganisms in a Free and Immobilized State and in Consortia with Bacteria

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Featured Application: The results obtained in this work may be of scientific and practical significance for the development of new biocatalytic approaches to the treatment of wastewater of complex composition, contaminated by perfluorocarbons and organophosphorus compounds, with simultaneous production of biomass of phototrophic microorganisms as renewable raw materials with a wide range of possibilities for conversion into valuable products. The results can also be taken into account for predicting changes in aquatic ecosystems when the mentioned pollutants enter them.

Abstract: The effects of the presence of perfluorocarbons (PFC) with a gas transport function in media with different phototrophic microorganisms on their growth rates and the accumulation of their biomass when using free and immobilized cells as inoculums were investigated. The significant increase in the average rate of biomass accumulation as well as levels of biomass accumulation in the presence of various PFCs were established for *Chlorella vulgaris* cells. When 1 g/L glycerol was introduced into the growth medium with PFCs and *C. vulgaris* cells, the increase in the rate of biomass accumulation was 9–32%. The maximum intracellular ATP concentrations corresponded to the combination of microalgae (*Chlorella vulgaris*) with bacterial cells (*Pseudomonas esterophilus* and *Rhodoccus ruber*) obtained with a mass ratio of 25:1. It provided for the formation of a consortium, which was able to accumulate the maximum amount of microalgae biomass for 3 days in the medium with PFCs and organophosphorus pesticide. The obtained data allow, on the one hand, predicting the growth of microalgae under environmental conditions in media with PFC pollution and, on the other hand, developing approaches to regulation of phototrophic microorganisms' growth in order to obtain and use their high biomass yields for various purposes.

Keywords: microalgae; fluoroorganic compounds; gas transport function; biomass accumulation; bacteria–microalgae consortium; methyl parathion degradation

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1. Introduction

The biomass of phototrophic microorganisms continues to be actively considered as a renewable resource that can be effectively used to produce various commercially significant products [1–3]. Unlike lignocellulose-containing waste, microalgae and cyanobacteria biomass does not require delignification before its deep conversion into biofuels [3–6], organic acids [7], compounds useful for the chemical synthesis of biodegradable polymers, commercially significant polysaccharides [8,9], etc. Commercial production of microalgae biomass is often limited by its relatively low cell productivity per unit volume in comparison with the growth rates of bacterial cells used in biotechnological processes [10].

To increase the economic profitability of the processes of obtaining and processing biomass of phototrophic microorganisms, they can be cultivated using wastewaters with different contents. The cell biomass cultivation can be combined with wastewater treatment and purification [3,11–13]. The prospects of using microalgae for the treatment of

wastewater of different compositions are due to the flexibility of their metabolism, which allows these cells to use inorganic and organic carbon sources, as well as their mixture. This allows the use of microalgae in the treatment of municipal, agricultural and industrial wastewater in various contexts, including tannery, textiles, paper, pharmaceuticals, food (coming from the production of tofu, dairy products, confectionery and alcoholic beverages, etc.), pig farms, etc. [14–17].

Among the wastewaters to be treated, those that contain organophosphorus compounds (OPCs) are often found in agriculture and chemical industries related to the production of pesticides. Usually, undecomposed residues of OPCs enter water systems and drains [18]. Perfluorocarbons (PFCs) are also actively used in industry, for example, in the production of cookware with non-stick coating, in water-repellent coatings in clothing and packaging, in fire-fighting equipment as well as in lubricants with a wide range of applications [19]. Due to their chemical and biological inertia, PFC enter the environment with waste and wastewater, where they can stay for a long time and gradually accumulate [20,21]. In contrast to OPCs, the presence of PFCs in river ecosystems is a relatively new problem for the environment [17], as it has been established that only some mycelial fungi and bacteria (mainly of the genus *Pseudomonas*) are able to decompose PFCs [19,22]. To date, the simultaneous presence of OPCs and PFCs has been determined in municipal wastewater [23–25], while their cumulative toxic effect on the human body has been noted [26].

Microalgae are common participants in natural consortia in various aquatic environments [27]. Therefore, when studying the possible influence of xenobiotics on living systems, they represent promising model objects. Today, the metabolic activity of microalgae and cyanobacteria in the presence of xenobiotics is being actively investigated due to the fact that toxic pollutants can be used by these microorganisms as sources of biogenic elements. Such widespread phototrophs as cyanobacteria of the genera *Nostoc* and *Arthrospira* (*Spirulina*), as well as microalgae of the genus *Chlorella*, can use OPCs as a source of phosphorus [28,29], released under the action of enzymes present in bacterial cells capable of degrading OPCs and, as a rule, coexisting under natural conditions with microalgae and cyanobacterial cells. To estimate the possibilities of implementing such coexistence, artificially created microbial consortia can be investigated.

Some PFCs and their derivatives belong to substances with a gas transport function, for which it is known that they actively carriage oxygen [30]. It is already known that the presence of chemically synthesized substances with a gas transport function in liquid media contributes to the accumulation of increased concentrations of microbial cell biomass [31,32]. In turn, the accumulation of biomass of phototrophic microorganisms is associated with their active consumption of carbon dioxide in aquatic environments. Because the solubility of carbon dioxide in water and in perfluorohexane [33] is significantly higher than the solubility of oxygen, it suggests that PFCs can have a significant intensifying effect on the process of cell growth of phototrophic microorganisms both under environmental conditions and during the implementation of appropriate biotechnological processes aimed at the quick accumulation of microalgae biomass.

Because bacteria among the various microorganisms have the most diverse set of metabolic processes, their combination with the biochemical potential of phototrophic microorganisms makes it possible to solve the problems of treating wastewaters of various compositions and origins as efficiently as possible. The detection of positive trends in the manifestation of interaction between bacterial and microalgae cells for removing xenobiotics is of great scientific and practical significance [34–40]. However, one of the important drawbacks of the biotechnological process is the possible presence of residual concentrations of toxic components of the treated wastewater (xenobiotics, heavy metals, etc.) in the accumulating microbial biomass [41]. This may limit its further use and therefore requires constant monitoring of its ecotoxicity and the intensification of wastewater treatment due to active cell growth. New knowledge in this direction can be used both to solve current environmental problems and to increase the efficiency of processes of

accumulating microalgae biomass in wastewater and for its further transformation into commercial products.

The aim of this work was to study the effect of the presence of different perfluorocarbon compounds with gas transport functions in environments with phototrophic microorganisms (taken in the form of individual cultures of microalgae or cyanobacteria, as well as in the form of participants in synthetically composed consortia with bacteria degrading OPCs) on the growth parameters and viability of microalgae and cyanobacteria cells.

2. Materials and Methods

2.1. Chemicals

Methyl parathion was acquired from Sigma-Aldrich (St. Louis, MO, USA). All other reagents for experiments were purchased from Chimmed (Moscow, Russia). The following perfluorocarbon compounds (PFC) were used in the studies: $CF_3(CF_2)_4CF_3$ (1,1,1,2,2,3,3,4,4,5,5,6,6,6-tetradecafluorohexane, perfluorohexane) from HaloPolymer, Moscow, Russia; and $C_{10}F_{18}$ (1,1,2,2,3,3,4,4a,5,5,6,6,7,7,8,8,8a-octadecafluorodecalin, perfluorodecalin) from Acros Organics, Geel, Belgium. In addition, $C_3F_7OCF(CF_3)CF_2OC_2F_5$ (3,6-dioxaperfluoro-5-methylnonane, polyether I) and $[C_3F_3O(C_2F_4CF_2O)_4C_2F_4]_2$ (4,7,10,13,16,19,22,25-octaoxaperfluoro-5,8,11,14,17,18,21,24-octamethyloctacosane, polyether II) were synthesized as described previously [32].

2.2. Microorganisms and Cultivation Condition

Cells of the phototrophic microorganisms *Chlorella vulgaris* C-1, *Arthrospira (Spirulina) platensis* (Nordst.) *Geitl. rsemsu* 1/02 and *Nostoc* sp. *rsemsu* Nss-14/11 were obtained from the IBCP RAS collection and the IPPAS collection of microalgae and cyanobacteria (Moscow, Russia). Bacterial cells of *Rhodococcus erythropolis* AC-1514D and *Pseudomonas esterophilus* V-1436D were obtained from the All-Russian Collection of Microorganisms (VKM, Moscow, Russia). The bacterial culture *Photobacterium phosphoreum* B-1717 was obtained from the Russian National Collection of Industrial Microorganisms (Moscow, Russia).

The preparation of the immobilized inoculums of *C. vulgaris* cells with and without combination into a bacterial consortium was carried out according to the methods described earlier [13,42]. Briefly, cells separated from the medium were suspended in a solution of poly(vinyl alcohol) (type16/1, 84 kDa, Sinopec Corp., Beijing, China). Then, this suspension was maintained at −70 °C using a DS 78 compact freezer (Dairei Asia Sdn. Bhd, Kuala Lumpur, Malaysia) for 3 days. Then, it was slowly defrosted via the following two stages: the first one, at −20 °C using a GN 3613 freezer (Liebherr, Biberach, Germany) for 3 h, and the second one, at 8 ± 2 °C in a 2201 Combicoldrac II refrigerator (LKB Instruments Haglund, Saffle, Sweden). The obtained samples of cells immobilized by inclusion into a polymer cryogel formed at subzero temperature were used in the further experiments.

Standard media (Tamiya medium, BG-11 medium, Zarrouk medium) [4] were used correspondently for biomass accumulation and cultivation of suspended microalgae (*Chlorella vulgaris*) and cyanobacteria cells (*Nostoc* sp. and *Arthrospira (spirulina) platensis*) when the cell suspensions were used as inoculums. Among investigated variants of media, one of them was used with the addition of 1 g/L glycerol.

Horticultural water from a gardening facility (Sovkhoz dekorativnogo sadovodstva, Moscow, Russia) was used for cultivation of immobilized inoculums of *C. vulgaris* cells and bacterial consortia. Wastewater with known characteristics [13] was applied in the experiments. It contained 0.99 g COD/L, where COD (chemical oxygen demands) was determined by the standard dichromate method [43]. The main chemical content of the wastewater was as follows: lipids—0.17 g/L; proteins—0.13 g/L; and carbohydrates 0.27 g/L, at a pH of 6.5.

The PFC additives in the growth medium were 0.5 or 1% (*v*/*v*). The initial concentration of methyl parathion was 0.15 mM in experiments with artificial consortia.

Cultivation of all microorganisms was carried out in an experimental laboratory-scale closed-type photobioreactor under the conditions described previously [44]. To implement

cultivation in a circulating regime for 3 days, cells of all phototrophic microorganisms were loaded into a photobioreactor with a 20 L working volume, into which a medium was pumped at a flowrate of 20 L/day through a system of glass tubes with an inner diameter of 35 mm and an outer diameter of 38 mm by means of a centrifugal pump. Carbon dioxide (99.8% *v*/*v*) was bubbled through the tubes around the clock through a silicone membrane at a rate of 1–2 mL/min. Also in this system, constant illumination of 5000 lux was provided by light sources with wavelengths in the intervals of 450–480 nm and 640–700 nm simultaneously. The photobioreactor was equipped with a fully automated system for monitoring the pH (7.2 ± 0.2) of the medium and controlling the cultivation temperature (22 ± 1 °C).

2.3. Biomass Separation and Mechanical Disintegration

Suspended biomass of *C. vulgaris* cells was separated from the culture medium by centrifugation (8000 rpm, 10 min, Avanti J25, Beckman, Brea, CA, USA). The disintegration of the separated biomass was carried out according to the method detailed in [45].

Mechanical disintegration of *C. vulgaris* microalgae biomass for the control of toxicity presence was carried out in a Mini-BeadBeater-24 ball mill (BioSpec Products, Bartlesville, OK, USA) (glass bead size 0.5 mm; rotor rotation speed 3000 rpm; in 0.5 mL cells, biomass loading was 80 mg DCW) for 3 min.

2.4. Analytical Methods and Calculated Parameters

Algae growth was controlled by cell counting using an improved Neubauer hemocytometer (Rohem Instruments, Nashik, Maharashtra, India) through an optical microscope (Biomed, Russia with a Biomed Lum 206070112209 nozzle and a Myscope 500 M digital camera for the microscope). Concurrently, the OD_{540} of the cell suspensions was controlled with an Agilent UV-8453 spectroscopy system (Agilent Technology, Waldbronn, Germany) to investigate the kinetic curve of growth. The calibration graphs reflected correlation between the OD_{540} and DCW of each culture of studied microorganisms used. The dry cell weight (DCW) of biomass was determined by a standard gravimetrical method using a sample drying at 105 °C to a constant weight.

The initial concentrations of suspended and immobilized cells of phototrophic microorganisms were 1.2 g dry cell weight/L.

For procedures of determination of the concentration of intracellular adenosine triphosphate (ATP) in microbial cells, methyl parathion and *p*-nitrophenol were used as published previously [42]. The pH value was determined as described previously [13].

The average rate of biomass accumulation ($V_{biomass}$) during the culture period was calculated from the equation: $V_{biomass}$ (g DCW/L/d) = $\Delta C/\Delta t$, where ΔC is the variation of biomass concentration (g DCW/L) within a cultivation time of Δt (days).

To control the presence of residual amounts of PFCs in culture media, the residual bioluminescence of immobilized photobacteria was determined when they were exposed to selected media samples. For this purpose, calibration dependences of the residual bioluminescence of photobacteria on certain concentrations of PFCs in the studied media were plotted and used. For this purpose the *Photobacterium phosphoreum* B-1717 cells with natural bioluminescence were cultured in a standard Fargaly medium. The accumulated biomass was immobilized by a known method [46] through its inclusion into a cryogel of poly(vinyl alcohol) so that its final concentration in the polymer matrix was 0.1%. The formed samples of immobilized photobacteria were used as sensitive elements for biosensor examination of the toxicity of the studied media. The estimations of toxicity of various media were carried out in a similar way as described earlier [46].

The data are shown as means of at least three independent experiments ± standard deviations (±SD). The statistical analysis was realized using SigmaPlot 12.5 (ver. 12.5, Systat Software Inc., San Jose, CA, USA). The significant ($p \leq 0.05$) differences between the obtained results were estimated by a one-way analysis of variance (ANOVA).

3. Results

3.1. Effect of Perfluorocarbon Compounds on the Growth and ATP Concentration of Phototrophic Microorganisms

The biomass accumulation and ATP concentration of the cells growing in media with PFCs were studied. The microalgae *C. vulgaris* C-1 and cyanobacteria (*A. (Spirulina) platensis* (Nordst.) Geitl. rsemsu 1/02 and *Nostoc* sp. *rsemsu* Nss-14/11) cells were investigated for 3 days under standard conditions for each microorganism when 0.5% or 1% (*v*/*v*) of a certain PFC (perfluorohexane, polyether I, perfluorodecalin or polyether II) was introduced into the cell growth medium (Table 1, Figure 1).

Table 1. Concentration of ATP (nmol/g DCW) * after 3 days of their cultivation in the culture media with various concentrations of different PFCs.

Microalgae	PFC (% *v*/*v*)								
	Control **	Perfluorohexane		Polyether I		Perfluorodecalin		Polyether II	
	0	0.5	1	0.5	1	0.5	1	0.5	1
C. vulgaris	33 ± 1	22 ± 1	24 ± 1	18 ± 0.9	26 ± 1	30 ± 1	24 ± 1	26 ± 1	22 ± 1
C. vulgaris ***	57 ± 2	32 ± 1	57 ± 2	37 ± 1	66 ± 3	57 ± 2	55 ± 2	62 ± 3	89 ± 4
Arthrospira platensis	27 ± 1	17 ± 1	18 ± 1	15 ± 1	21 ± 1	26 ± 1	20 ± 1	20 ± 1	18 ± 1
Nostoc sp.	63 ± 3	40 ± 2	41 ± 2	33 ± 1	50 ± 2	60 ± 3	44 ± 2	48 ± 2	42 ± 2

* The initial concentration of ATP (nmol/g CDW) was as follows in the cells: it was 11 ± 1 in *C. vulgaris*, 19 ± 1 in *A. platensis* and 59 ± 1 in *Nostoc* sp.; ** Growth was without PFCs; *** Cultivation was with the addition of 1 g/L of glycerol to the culture medium.

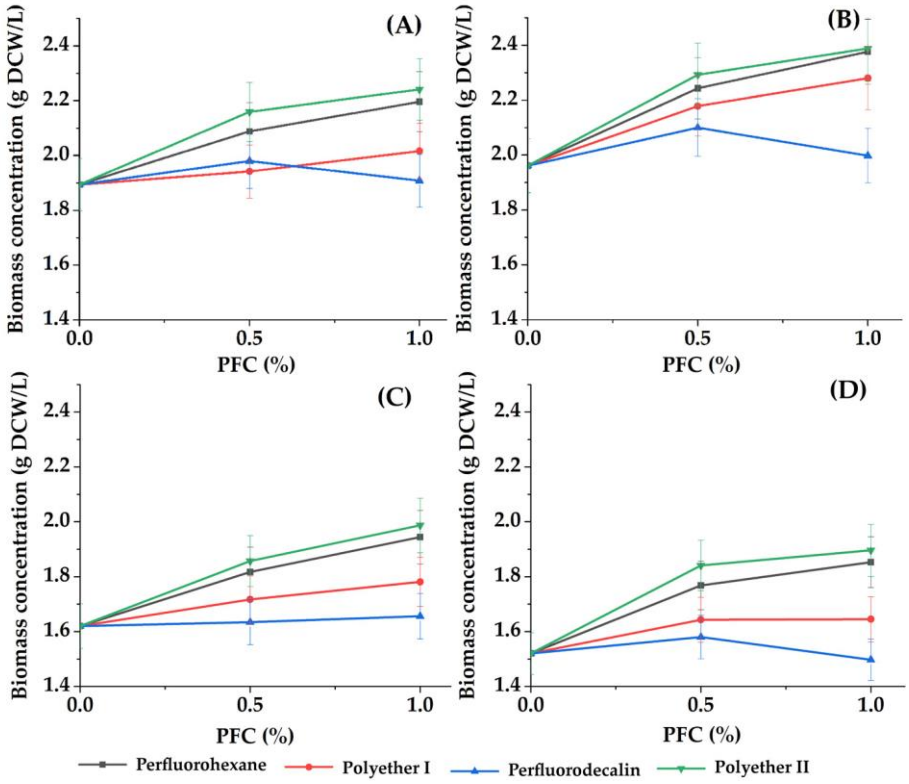

Figure 1. Dependence of the concentration of accumulated biomass of phototrophic microorganisms ((**A**) *C. vulgaris* cells in Tamiya medium, (**B**) *C. vulgaris* cells in Tamiya medium with 1 g/L glycerol, (**C**) *A. platensis* cells in Zarrouk medium, (**D**) *Nostoc* sp. cells in the medium BG-11) on the concentrations of different PFCs. Control: the concentration of accumulated cell biomass of phototrophic microorganisms when concentration of PFC was 0.0%.

The concentration of the suspended cell inoculum was the same in all variants of cells (1.2 g DCW/L), and it was high enough compared to most of the known studies [30,47,48]. The choice of such a cell concentration was based on the data previously obtained by the authors [13], when it was revealed that a high rate of cell growth of phototrophic cultures can be achieved in a short period of time with a high density of the cell population in the inoculum. High cell population density and growth rates of phototrophs, among other things, stimulated the process of "blooming" of the reservoirs. Because the rate of biomass accumulation began to decrease after 3 days, experiments were carried out for no more than 3 days in order to analyze the process according to the initial growth rates.

For all the studied cultures, a positive effect was noted from the presence of perfluorohexane, polyether I or polyether II in the growth medium, manifested in an increased average rate of biomass accumulation in comparison to the parameter value in the control (without PFC) (Figures 1 and 2).

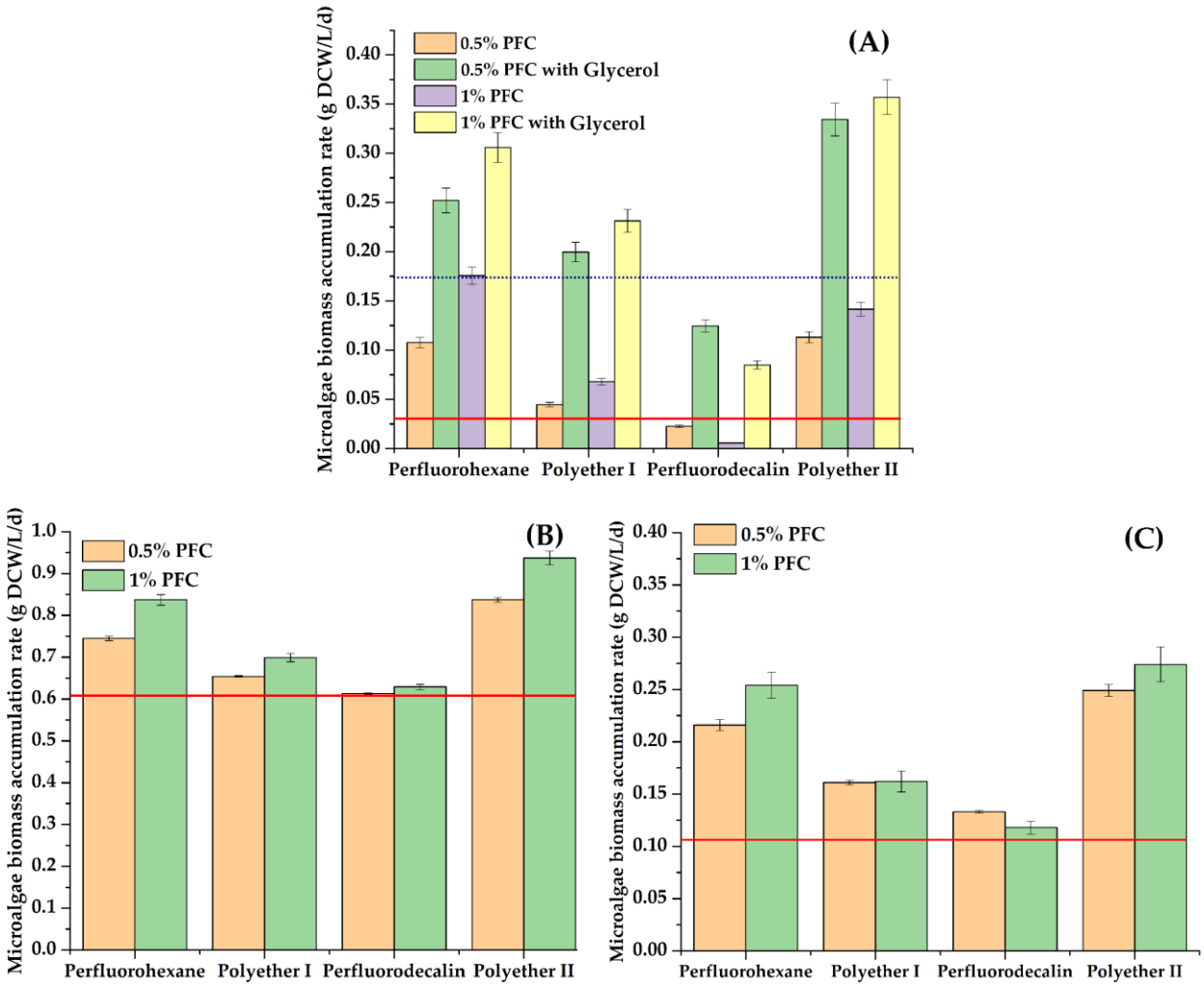

Figure 2. The average rate of biomass accumulation (g DCW/L/d) for 3 days: *C. vulgaris* cells in Tamiya medium (**A**), *A. platensis* cells in Zarrouk medium (**B**), *Nostoc* sp. cells in BG-11 medium (**C**). Controls: results of cultivation of cells without PFC and with 1 g/L glycerol are marked by a blue dotted line (**A**), and results of cultivation without both PFCs and glycerol are marked by a red line (**A**–**C**).

The most pronounced effect was observed when perfluorohexane or polyether II was introduced into the growth medium. These results were generally consistent with the data obtained earlier regarding the accumulation of *E. coli* cell biomass in the presence of PFCs [31].

The maximum and lowest values of average rate of biomass accumulation were found for *A. platensis* and *Nostoc* sp. cells, respectively (Figure 2). In a standard growth medium without additional additives, an increase in the concentration of perfluorohexane, polyether I or polyether II by 2 times from 0.5% to 1% (*v*/*v*) was accompanied by a slight increase (up to 8%) in the concentration of the accumulated biomass of each of the studied phototrophs.

It was noted that by 72 h of cultivation in the presence of a PFC, the level of intracellular ATP concentration in phototrophic cells decreased slightly under experimental conditions (Table 1).

It was found that when 1 g/L glycerol is introduced into the growth medium with *C. vulgaris* cells as an additional component for mixotrophic nutrition, an increase in the rate of biomass accumulation by 9–32% is possible. This effect was most clearly expressed when glycerol was combined with polyether I (Figure 1). This fact suggests that the replacement of the Tamiya medium with wastewater containing an organic food source will contribute to an increase in the rate of biomass accumulation of *C. vulgaris* microalgae cells.

Analysis of Figure 2A suggests that in the case of simultaneous ingress into water bodies of sources of heterotrophic nutrition (organic substances) and substances with a gas transport function, a particularly intensive growth of phototrophic microorganisms should be expected, which is important for forecasting the development of ecosystems.

C. vulgaris microalgae cells were selected for further studies, as they are most often used as participants in biosystems for wastewater treatment, and they can also be present in fresh and salty reservoirs and in soil; they can also be part of aerobic active sludge [49].

Commercial perfluorohexane, which is already being produced in industry and is actively used in various fields as a refrigerant, fire-extinguishing agent, component of dielectric media, solvent and foam-blowing agent [50], and polyether II were used as PFCs from among the studied ones, as in the presence of this PFC, the maximum rates of accumulation of phototrophic biomass were noted.

3.2. Effect of PFC on the Characteristics of Chlorella Biomass Accumulation in Presence of Immobilized Inoculum under Conditions of Periodic Cultivation in Wastewater

The toxicity analysis of the studied PFCs, carried out using bioluminescent bacterial cells, confirmed the presence of toxicity in all media with a content of 1% (*v*/*v*) PFC. The level of bioluminescence in cells exposed in the analyzed media samples decreased by 20–97% for 0.5 h depending on the type of PFC. Perfluorodecalin turned out to be the most toxic for these cells. Despite the fact that PFCs and their derivatives are actively used in medicine, the presence of cytotoxicity with PFCs is generally consistent with the literature data [51–53]. The unique properties of the C-F bond and F-F interactions in PFC structures require increased attention to such compounds in the direction of minimizing the possibility of their ingress into the environment [53]. However, such a negative effect can manifest itself in relation to certain microorganisms, according to the data obtained with phototrophs (Figures 1 and 2), and bioluminescent cells were used for the current control of changes in the toxicity of the media implemented in the work because they are sensitive to PFC presence at a certain concentration (Figure S1).

To study the process of accumulation of suspended biomass of *C. vulgaris* microalgae, an immobilized inoculum was used as a concentrated form of stabilized cells and as a model of a self-stabilizing ecosystem that can be formed naturally by the phototrophs. This inoculum was introduced into horticultural water from a gardening facility used as medium for cell cultivation. Previously, it was established that this is a good enough medium for the cultivation of *C. vulgaris* cells [13].

The immobilized inoculum has undoubted advantages when used for wastewater treatment, as it is easily separated from the culture fluid, and cells in this form show resistance to variations in the chemical composition of wastewater and its physical properties [13,54]. The liquid medium was drained with the accumulated free cell biomass from the photobioreactor every 3 days. The accumulated biomass of suspended cells was

separated from the medium and analyzed, and the medium with the addition of glycerol was loaded back into the photobioreactor (Figure 3).

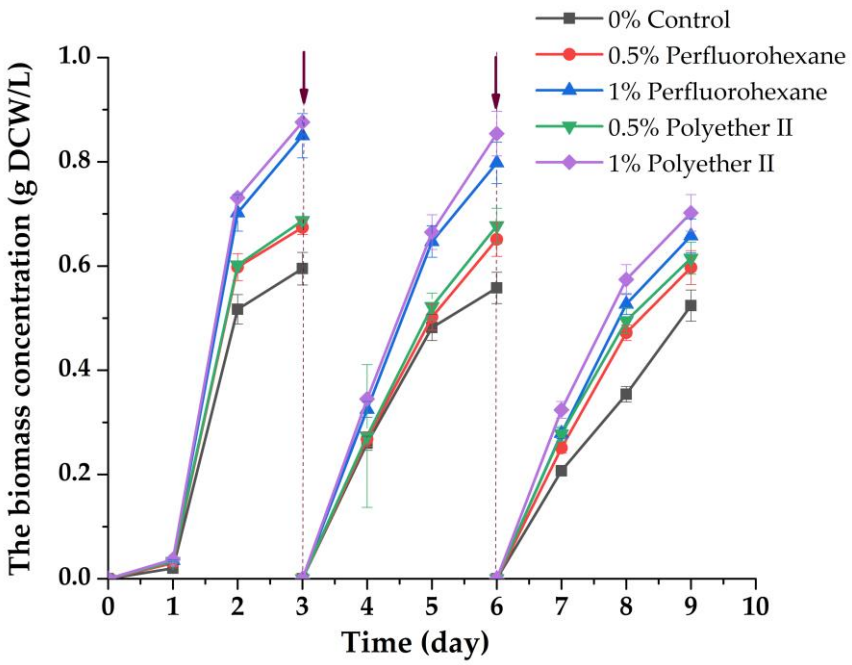

Figure 3. Changes in the concentration of *C. vulgaris* suspended biomass during its accumulation in wastewater with or without addition of PFCs, using an immobilized inoculum. The arrows indicate the time of the following procedure: draining of the liquid medium with suspended biomass; separating biomass of free microalgae cells; adding 1 g/L glycerol to the waste medium; returning of the culture liquid medium to the photobioreactor with the immobilized inoculum.

For all the studied variants, in the presence of PFCs at the end of each cycle, the biomass increased the level of the same parameter revealed for cells in the control, where PFCs were absent. The maximum excess of the level of biomass accumulation as compared to the control was about ~25% (Figure 1). The 10.5–12% decrease in the rate of free cell biomass accumulation in the control was comparable with a decrease in the rate of microalgae cell biomass accumulation in the medium with 0.5% (v/v) PFC for three cycles of using an immobilized inoculum. The decrease in the rate of biomass accumulation was 20–23% for three working cycles when 1% (v/v) PFC was introduced into the media.

Thus, it was found for the first time that a significant increase in the levels of biomass accumulation of free microalgae cells should be expected when they enter by case or are directly loaded into wastewater containing different types of PFCs. At the same time, the state of the inoculum of *C. vulgaris* cells (in the form of a suspension or a sample of cells immobilized in porous gel structures), as it turned out, does not change the overall growth trend, which exceeds the control if a PFC is present in the cell culture medium.

At the end of the process (at the ninth day of immobilized cell cultivation), the toxicity of the culture medium was assessed, and it was found that it remained at the initial level. The suspension biomass of *C. vulgaris* cells was separated from the liquid and disintegrated. Furthermore, the toxicities of both the spent broth and disintegrated microalgae biomass were separately evaluated with bioluminescent bacteria. It was found that the biomass is absolutely non-toxic, while the liquid medium used for cell cultivation maintained the same initial level of toxicity due to the initial introduction of PFCs to it. In this regard, it seems appropriate in the case to implement similar conditions for the biotechnological process of biomass accumulation of phototrophic microorganisms: the use of a closed-type photobioreactor with the returning of spent culture liquid medium containing PFCs to the production cycle with the addition of a fresh portion of a carbon

source for heterotrophic nutrition of cells at a new stage of biomass cultivation with an increased level of accumulation, but without intoxication. This can be an important point for the practical use of such biomass in obtaining commercial products. Actually, glycerol-containing wastes coming from the treatment of the same microalgae biomass in some commercial products can be used as such carbon additives to the photobioreactor. It is known that this is possible [55,56].

3.3. Effect of Joint Presence of PFCs and OPCs on Consortia of Bacterial and Microalgae Cells

As already noted, PFCs and OPCs are present in urban wastewater, as well as in the wastewaters of enterprises using them [23–25]. Among other things, these substances are also contained in dust, which can enter water bodies together with precipitation.

Microalgae, especially of the genus *Chlorella*, are actively used for wastewater treatment, including as parts of consortia. Wastewater is also offered as a medium for the accumulation of phototrophic biomass, where other microorganisms can be cultivated in parallel. It is known that *N*-acyl homoserine lactones (AHL) produced by Gram-negative bacterial cells stimulate the growth of microalgae, in particular those of the genus *Chlorella* [57,58]. On the one hand, this information sets a new direction in the search for ways to increase the productivity of the processes of directed accumulation of phototrophic biomass. On the other hand, it causes concern from the point of view of uncontrolled development of bacterial–algal consortia in environmental objects.

In this regard, it is necessary to investigate the process of accumulation of biomass of microalgae of the genus *Chlorella* in association with bacterial cells when using wastewater containing PFCs and OPCs simultaneously as a nutrient medium. Such a study simulates a situation that may take place in real ecosystems, as xenobiotics can enter water bodies, which helps accelerate the accumulation of phototrophic biomass. The presence of other microorganisms can lead to even more intensive cell growth.

To study changes in the rate of accumulation of *Chlorella* microalgae biomass in the presence of bacteria capable of producing AHLs, three synthetic consortia were formed. At the same time, they were obtained by joint immobilization of microalgae biomass and a bacterial consortium at different ratios (Table 2). A previously developed immobilized artificial consortium was based on microorganisms belonging to Gram-positive (*R. ruber*) and Gram-negative (*P. esterophilus*) bacterial cell types and was capable of jointly performing rapid and efficient degradation of OPCs [42].

Such a combination of components, in our opinion, should ensure the presence of bacterial AHLs in close proximity to *C. vulgaris* cells, as well as facilitate gas exchange (O_2 and CO_2) between microorganisms. Among other things, such an immobilized consortium is a kind of nature-like model, as bacterial cells and phototrophic microorganisms coexist in water bodies of the environment, forming self-immobilizing biosystems (biofilms, floccules, mats), as well as being based on natural solid organic–mineral particles present in ecosystems.

The study of changes in the rate of accumulation of microalgae biomass in the presence of bacteria was carried out in wastewater with the simultaneous presence of a PFC (perfluorohexane or polyether II) at different initial concentrations and an OPC (methyl parathion). It was found that in all the studied variations (Table 2), after 20 h, the degree of degradation of methyl parathion in the medium was 100%. Previously, for a bacterial consortium based on *P. esterophilus* V-1436D and *R. ruber* AC-1513D cells with a mass ratio of 2:1 [42], a similar result with the same concentration of methyl parathion was achieved in 24 h [42]. In this work, the joint immobilization of bacterial cells and microalgae cells probably provided constant oxygen access to bacteria and the removal of carbon dioxide from them by microalgae cells, which provided intensification of the degradation process of methyl parathion.

Table 2. Values of study parameters established after 72 h of cultivation of an immobilized consortium of *P. esterophilus* bacteria with *R. ruber* and *C. vulgaris* microalgae in wastewater with 0.15 mM methyl parathion.

Studied Parameter	Biomass Ratio "Microalgae:Bacteria"					
	6:1	**12:1**	**25:1**	**6:1**	**12:1**	**25:1**
	Perfluorohexane			**Polyether II**		
	0% *v*/*v*					
$ATP_{in\ granules}$ (nmol/g)	0.4 ± 0.0	0.8 ± 0.0	15.2 ± 0.7	0.4 ± 0.0	0.8 ± 0.0	15.2 ± 0.7
$ATP_{in\ medium}$ (nmol/mL)	33 ± 0.1	37 ± 1	80 ± 3	33 ± 0.1	37 ± 1	80 ± 3
The average rate of biomass accumulation (g DCW/L/d)	0.25 ± 0.0	0.35 ± 0.0	0.41 ± 0.0	0.25 ± 0.0	0.35 ± 0.0	0.41 ± 0.0
	0.5% *v*/*v*					
$ATP_{in\ granules}$ (nmol/g) *	0.8 ± 0.0	4.3 ± 0.2	17.7 ± 0.9	0.8 ± 0.0	4.32 ± 0.21	17.7 ± 0.9
$ATP_{in\ medium}$ (nmol/mL)	34 ± 1	38 ± 1	87 ± 4	35 ± 1	38 ± 1	88 ± 4
The average rate of biomass accumulation (g DCW/L/d)	0.32 ± 0.01	0.36 ± 0.01	0.44 ± 0.02	0.3 ± 0.01	0.35 ± 0.01	0.48 ± 0.02
	1% *v*/*v*					
$ATP_{in\ granules}$ (nmol/g) *	1.5 ± 0.1	2.5 ± 0.1	19.8 ± 0.9	1.6 ± 0.1	2.5 ± 0.11	22.7 ± 0.8
$ATP_{in\ medium}$ (nmol/mL)	34 ± 1	38 ± 1	89 ± 4	35 ± 1	40 ± 2	90 ± 4
The average rate of biomass accumulation (g DCW/L/d)	0.36 ± 0.01	0.38 ± 0.01	0.53 ± 0.02	0.37 ± 0.01	0.37 ± 0.01	0.54 ± 0.02

* Initial concentration of $ATP_{in\ granules}$ was 4.1 ± 0.2 nmol/g.

From the data obtained (Table 2), it followed that the ratio of the biomasses of different microorganisms in the composition of the pellet affects the level of concentration of intracellular ATP of immobilized and suspended cells. The maximum ATP values corresponded to the combination of "microalgae:bacteria" at a ratio of 25:1, which, apparently, provides for the formation of a consortium with intercellular interaction of cultures. The combination of microalgae with a bacterial consortium was expected to increase the rate of accumulation of phototrophic biomass. It was possible to accumulate the maximum amount of microalgae biomass in 3 days in the medium when the ratio between participants of the microbial combination "microalgae:bacteria" was 25:1.

The presence of a PFC in the medium, as before, had a positive effect on the rate of accumulation of microalgae biomass. It was noted that, unlike individual cultures, when microalgae were combined with bacteria, there was no decrease in ATP concentration in the presence of PFCs as compared to the control (Tables 1 and 2). Perhaps this effect was due to the formation of mutualistic relations between the participants in microbial societies, manifested in bacterial and microalgal synergy for removing xenobiotics and environmental toxicity. Probably, AHLs synthesized by bacteria contributed to the enhancement of the photoautotrophic growth of *C. vulgaris* cells, which is generally consistent with the literature data [57].

Evaluation of the intensity of photobacteria bioluminescence after their exposure in culture media containing 0.15 mM methyl parathion and different concentrations of PFCs (Table 3) showed that changes in the residual bioluminescence of cells during the first 24 h already indicated a tendency toward decreasing toxicity in the media.

Table 3. Residual bioluminescence * (%) of photobacteria in media after 24 h of cultivation of a consortium of *P. esterophilus*, *R. ruber* bacteria and *C. vulgaris* microalgae in wastewater with 0.15 mM methyl parathion and different PFCs.

PFC (% *v*/*v*)	Biomass Ratio "Microalgae:Bacteria"					
	6:1	12:1	25:1	6:1	12:1	25:1
	Perfluorohexane			Polyether II		
0	78.1 ± 3.7	89.1 ± 4.1	92.7 ± 4.3	80.4 ± 2.5	95.1 ± 4.1	95.7 ± 3.3
0.5	6.8 ± 0.3	8.0 ± 0.3	22.4 ± 1.1	6.4 ± 0.2	8.3 ± 0.3	20.4 ± 1.1
1	3.0 ± 0.1	6.0 ± 0.2	15.0 ± 1.7	2.0 ± 0.1	5.0 ± 0.1	14.0 ± 1.7

* Initial indicators of residual bioluminescence of cultivation media before the introduction of methyl parathion—100% (0% PFC), 6.6–10.3% (0.5% PFC), 2.2–4.1% (1% PFC); after the introduction of methyl parathion—75.2–75.5% (0% PFC), 5.8–9.4% (0.5% PFC), 2.0–3.8% (1% PFC).

After 72 h of cultivation, the residual bioluminescence of photobacteria was almost 100%. This is explained by the fact of complete decomposition of methyl parathion and the utilization by cells of its biodegradation products. As for PFCs, they can be partially utilized by *P. esterophilus* bacterial cells because it is known that individual bacterial strains of the genus *Pseudomonas* are able to use PFCs as a nutritional source (degradation of 1 g/L PFC can reach 75%) [59]. Based on the above, for the treatment of wastewater containing PFCs, it is possible to recommend the use of artificial consortia of microorganisms containing *C. vulgaris* microalgae and *Pseudomonas* bacteria, which are effective destructors of a number of xenobiotics.

4. Discussion

As one of the approaches to the intensification of the growth of phototrophic microorganisms, their co-cultivation with bacteria can be considered. For instance, the co-cultivation was effective in the treatment of wastewater when the following microbial partners were joined in the same biosystem: *Chlorella vulgaris* and *Bacillus subtilis* [60]; *Pseudomonas* and *Cyanobacteria* cells [17]; *Chlorella vulgaris*, *Ettlia* sp. and *Chlamydomonas reinhardtii* [61]; *Chlorella* sorokiniana and wastewater bacteria; *Auxenochlorella protothecoides* and wastewater bacteria; *C. vulgaris* and *Pseudomonas putida*; *C. vulgaris* and *Bacillus licheniformis*; Desmodesmus sp. and nitrifying bacteria; *C. sorokiniana* and denitrifying bacteria; *C. vulgaris*, *Ettlia* sp., *C. reinhartii* and hydrogen consumer denitrifiers; and *Chlorella*, Klebsiella and Acinetobacter cells [39]. Additionally, co-cultivation biosystems are used for commercialization of the microalgae biomass accumulation process [35]. Bacteria synthesizing AHLs are able to regulate the quorum sensing (QS) of microalgae and cyanobacteria. Consortia of bacteria and phototrophic microorganisms are widespread. In nature, they are formed as a result of the symbiotic interaction of microorganisms: microalgae and cyanobacteria cells produce oxygen, which is used by aerobic bacterial cultures that emit carbon dioxide, which phototrophs need. The presence of compounds with a gas transport function in the environment, which appear in the environment as pollutants, in this case can have a positive effect on the structure of mutually beneficial relationships between microorganisms. Studies on this topic are not yet known, but the matter is relevant today. It is possible to obtain new information in this direction by studying synthetic consortia; however, today algal–bacterial synergy is already used in the development of synthetic consortia for the destruction of various xenobiotics [62].

Recently, studies on the influence of the QS mechanism, manifested in the ability of bacterial cells to interact with each other through signaling molecules and to influence algae–bacteria interactions, have been of particular interest [63]. To date, it has been revealed that there are a number of AHL molecules, as well as other lactone-containing QS signaling molecules with different chemical structures and products of their destruction, which can have different effects on microalgae cells [64]. Under certain conditions, the presence of a keto group in the structure of an AHL molecule can lead to its enzyme-free rearrangement and the formation of tetramic acids, which can have a significant algicidal

effect on the growth and photosynthesis of individual microalgae [65]. However, it is known that AHLs secreted by bacteria can bind to microalgae cells and contribute to their self-aggregation and stabilization [66]. Previously, it was found that in the combined systems of algae–bacteria, an excess of AHLs can restrain the initial growth of *Chlorophyta* sp., thereby having a negative effect on the characteristics of microalgae [67]. The results obtained in this work clearly indicate that the variants of combinations of bacteria and *Chlorella* that were experimentally created in this study turned out to be successful, and this should be taken into account by researchers who work in this field. The concentration of intracellular ATP is a marker of the metabolic activity of cells and the level of their viability [13]. The analysis of this biochemical characteristic of cells by bioluminescent ATP-metry makes it possible to quickly assess with high selectivity and sensitivity the effects of various conditions of cultivation of various cells on their functional activity [68]. In this work, data on the concentration of intracellular ATP were used to monitor the state of cells, both in the presence of xenobiotics and in the formation of consortia of *Chlorella* cells with bacteria. ATP-metry proved to be indispensable, especially when assessing the state of immobilized cells in granules (Table 2), as it was impossible to isolate them without damage. It was found that an increase in the level of ATP in the analyzed cell samples clearly corresponds to an increase in the level of accumulated biomass in them. Such an analysis of cellular systems by two parameters (ATP and biomass accumulation) allowed us to discuss the positive relationships that developed between cells of different cultures as a part of formed artificial consortia when the cells were combined at certain ratios.

The analysis of the results obtained in this work suggests that if PFCs enter various water bodies, we should expect an intensification of the growth of some microorganisms, in particular a number of microalgae and primarily *C. vulgaris* microalgae cells. The question remains open about the possibility of using PFCs in biotechnological processes to intensify the growth of microalgae. In our opinion, special use of chemically and biologically inert substances should be carried out in closed systems to avoid their accumulation in the environment.

Interestingly, the ability of some phototrophic microorganisms to utilize OPCs was recently explained as a possible result of the plasmid transfer of necessary genes from bacteria due to the co-existence of microalgae and bacterial cells in native consortia [69–72]. Thus, the results obtained in this work have practical significance for researchers in the field of developing new biocatalytic approaches to the treatment of wastewaters of complex composition containing OPCs and PFCs while simultaneously obtaining valuable renewable raw materials—biomass of phototrophic microorganisms.

Additionally the results of this work indicate that with a combination of factors such as the presence of various nutrition sources and the presence of PFCs in the medium of microalgae and bacteria capable of regulating the QS of phototrophs, an active increase in microalgae biomass can be registered in environmental objects, which can change the equilibrium in such ecosystems. A recent study of the interaction of PFCs and microorganisms present in the aquatic environment showed that green microalgae *Desmodesmus subspicatus* cells are not capable of growing in presence of perfluorohexane, perfluorooctane or perfluorodecane phosphonic acids [73]. For these microorganisms, a decrease in the intensity of green coloration in the presence of PFCs was noted, which indirectly indicates a negative effect of PFCs on microalgae chlorophyll, resulting in cell fluorescence being lost. The fact that *Chlorella* cells were completely resistant to the possible negative effects of PFCs in this study is significant for predicting the viability of these microalgae in vivo under similar conditions.

5. Conclusions

The conditions associated with an increase in the rate of accumulation of biomass of various phototrophic microorganisms by several times compared with their control variants were investigated. It was shown for the first time that the presence of a number of PFCs in the medium significantly stimulates the growth of phototrophs present in the studied

media in the form of individual cultures or in combination with a bacterial consortium of soil bacteria of the genera *Pseudomonas* and *Rhodococcus*. It turned out that due to cell synergy in wastewater with PFCs and OPCs under the action of a synthetic immobilized consortium based on *C. vulgaris* cells with *P. esterophilus* and *R. ruber*, it is possible to carry out 100% degradation of methyl parathion and obtain intensive accumulation of biomass of free microalgae cells. The study of the vital activity of phototrophic microorganisms and their consortia that are characteristic of water bodies, with the simultaneous presence of OPCs and PFCs in them, has not been carried out by anyone before. The results of the presented experiments make it possible to predict the likely changes in such a system and, in general, indicate the need to assess the possible ecotoxicity of such environments.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app13031868/s1, Figure S1. Residual intensity of bioluminescence of photobacterial cells dependent on the concentrations of different perfluorocarbon compounds in the medium for bacteria exposition.

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