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Identifying Key Environmental Indicators in the Assessment of the Proof-of-Concept in Pigment Production from the Marine Cyanobacterium *Cyanobium* sp.

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Abstract: Cyanobacteria have long attracted market interest as a source of natural compounds such as pigments with proven bioactivity (carotenoid and phycobiliproteins). The cultivation and extraction processes for such compounds have been developed at different levels, from laboratory trials to photobioreactors on a demonstration scale. Based on this experience, it is possible to propose how the different stages of the process can be improved based on environmental performance indicators. The Life Cycle Assessment (LCA) methodology allows to identify the hotspots that represent the greatest environmental impacts and to propose strategies to focus on those stages that can be improved. The general environmental indicators have been identified and the results showed that cyanobacteria cultivation has the greatest influence on environmental impact for all scales considered (from 20 L to 100 m^3), which is attributed to the energy requirements. The main changes proposed to reduce the impact should focus on the stages of reactor cleaning, culture medium sterilisation and biomass drying. The implementation of these improvement alternatives can reduce the impact of the production and extraction processes by 85%. This work demonstrates how technological development must go hand in hand with impact assessment to make the best decisions in the overall process.

Keywords: environmental assessment; life cycle assessment; carotenoids; phycobiliproteins; process scale-up

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1. Introduction

Cyanobacteria are the only known prokaryotic organisms capable of oxygenic photosynthesis, making them a unique class of organisms. Many ecosystems rely on cyanobacteria for carbon dioxide fixation and oxygen release, making these organisms key primary producers in the trophic chain [1]. As producers of secondary metabolites, such as mycosporine-like amino acids, alkaloids, amides, fatty acids and peptides, cyanobacteria are a valuable source of natural products [2]. Because they are photosynthetic organisms, cyanobacteria synthesise a variety of pigments (chlorophylls, carotenoids and phycobiliproteins). Due to their colour and bioactive characteristics, these pigments are well known for their commercial application in food, feed, nutraceuticals, cosmetics, and pharmaceuticals [3,4]. Considering the content in pigments, they have become the components with the highest market growth potential for microalgae producing companies [5]. On the

other hand, the biotechnological potential of cyanobacteria has not yet been fully exploited. It is estimated that there are more than 100,000 species [6], but only a few thousand strains are preserved in culture collections and only a minority have been produced on a large scale [7].

To ensure pigment production by cyanobacteria on an industrial scale, the current gap in technical scale-up to produce higher biomass yields at reduced production costs must be overcome [8,9]. This problem is intensified by the fact that most research is carried out on a small scale, with technologically complex equipment where scale-up is a challenging step. Consequently, industrial-scale processes based on cyanobacteria still present limitations in biological, engineering, and economic terms [8,9].

The present study focused on the marine cyanobacterium *Cyanobium* sp. LEGE 06113 isolated in northern Portugal. It is unicellular with a small size (approx. 1 μm), it does not form biofilms, and is easily harvestable by centrifugation. Its potential was first proposed as a producer of some bioactive compounds, such as hierridin B and C, with antitumour and antimalarial activities [10–12], however, the content of such compounds is extremely low, which reduces its interest in favour of the synthetic route [13]. On the other hand, the production and extraction processes of pigments with antioxidant and anti-inflammatory properties have been investigated [14–16]. Specifically, the process was optimised by focusing on two bioactive pigment extracts from the same biomass, one targeting carotenoids and the other phycobiliproteins. Both pigment extracts can be used in the cosmetic industry, especially considering the short range of species approved for food or nutraceuticals in Europe. However, all studies with this cyanobacterium have been conducted on a small scale (maximum 20 L).

To assess the potential development of a large-scale production process, it is necessary to apply process modelling and software-based tools to estimate the main process flows and thus carry out a preliminary techno-economic assessment. In the framework of cyanobacteria-based production processes, Lopes et al. [17] evaluated direct ethanol production using a genetically modified *Synechocystis* sp. in a 100 m^3 reactor. Other studies on pigments techno-economics can be found for microalgae, including astaxanthin from *Haematococcus pluvialis* [18], β-carotene from *Dunaliella salina* [19], and a carotenoid-rich extract from *Chlorella vulgaris* [20]. Biorefineries routes based on a techno-economics analysis were also reported for pigment production, such as the one proposed for *D. salina* and *H. pluvialis* by Thomassen et al. [21], and comprehensively reviewed by Thomassen et al. [22] and by Banu et al. [23].

Based on the results of the techno-economic analysis, it is possible to compile the inventory data needed to carry out an environmental analysis, so that the list of inputs, raw material and energy requirements, and process outputs, comprising products, by-products and emissions, are extrapolated into environmental impacts associated with the entire life cycle of a product or process [24]. This is the aim of the Life Cycle Assessment (LCA) methodology, which highlights the environmental problems associated with the entire value chain of a product or process, avoiding transferring the environmental burden from one environmental compartment to another [25,26].

There are several references reporting the application of LCA to assess environmental impacts focusing on marine organisms and their compounds [27]. Few LCA studies of cyanobacteria have been conducted, limited to *Arthrospira* spp. and mainly focused on biofuels [28–31]. In the specific context of pigment production, Papadaki et al. [32] used LCA as a tool for solvent selection for phycocyanin extraction from *Arthrospira platensis*, and Käferböck et al. [33] described the use of pulsed electric fields as a pre-treatment for phycocyanin recovery. Thus, no information on LCA research on *Cyanobium* sp. is available.

This report includes the environmental assessment of the obtaining of bioactive pigments from cyanobacteria by applying the LCA methodology. The simulation tool SuperPro Designer® has been used to carry out the process modelling to obtain the necessary data for the compilation of the life cycle inventories based on the reported laboratory data.

Once the environmental profiles have been obtained, it is possible to identify the main hotspots of the process, i.e., the stages and/or materials that contribute most to the environmental loads and subsequently carry out a sensitivity analysis to improve and optimise the evaluated scenario.

2. Materials and Methods

2.1. Goal and Scope

The *Cyanobium*-based process produces two different high-value products, a carotenoid-rich extract, and a phycobiliprotein-rich extract. The environmental impact was estimated for single batch operation (one cycle of production) at different scales: 20 L, 140 L and 100 m^3, but also considering the same functional unit for all three scenarios: one litre of culture broth. In addition, both mass and economic allocation approaches have also been considered, as two products are obtained: carotenoid and phycobiliproteins extracts with different market values.

2.2. Process Description

The carotenoid and phycobiliprotein production process was divided into seven subsystems with the aim of maximising biomass utilisation and to reduce product wastage. The evaluated system and its boundaries are depicted in Figure 1.

(S1) Pre-inoculum: first, the reactor was cleaned using sodium hypochlorite. Then, BG11 culture medium [16,34] was prepared for 10% of the final volume of the batch, and heat-sterilised prior to the addition of the *Cyanobium* sp. LEGE 06113 inoculum. The cyanobacterium culture is incubated for seven days under controlled conditions (20 °C; aeration: 0.75 vvm; illumination: 40 W m^{-2}, white LEDs and light: dark cycle of 16:8 h).

(S2) Cultivation: first, the reactor was cleaned using sodium hypochlorite. Then, a BG11 culture medium was prepared for 90% of the final volume of the batch, and heat-sterilised before the addition of the pre-inoculum. The culture was prolonged for 14 days (10 days on white LED plus 4 days on red LED) under the same conditions as the pre-inoculum. The final biomass concentration was 2 g L^{-1}.

(S3) Harvesting: Biomass was harvested by centrifugation and concentrated 100 times to a final concentration of 200 g L^{-1}.

(S4) Dewatering: The dewatering of the biomass was performed by freeze-drying (48 h).

(S5) Extraction I: Carotenoids were extracted using ohmic heating technology and ethanol (99% + NaCl 0.2%) as solvent at a ratio of 10:1 to biomass [14]. The mixture was centrifuged, and the ethanol was separated in a rotary evaporator, obtaining the dry carotenoid extract (0.27 g g^{-1} of dry biomass).

(S6) Extraction II: Phycobiliproteins were extracted from the remaining biomass, which was resuspended in water (at the same volume as ethanol) and shaken for 30 min. The solution was centrifuged and freeze-dried (48 h). At this point, the phycobiliproteins extract was obtained (0.25 g g^{-1} of dry biomass).

(S7) Recovery of residual biomass: The remaining biomass was dried in a drying oven, yielding a residual biomass product (0.48 g g^{-1} of dry biomass). The dry residual biomass is produced with the aim to be used as a fertiliser by-product due to its high nitrogen content (12.1%).

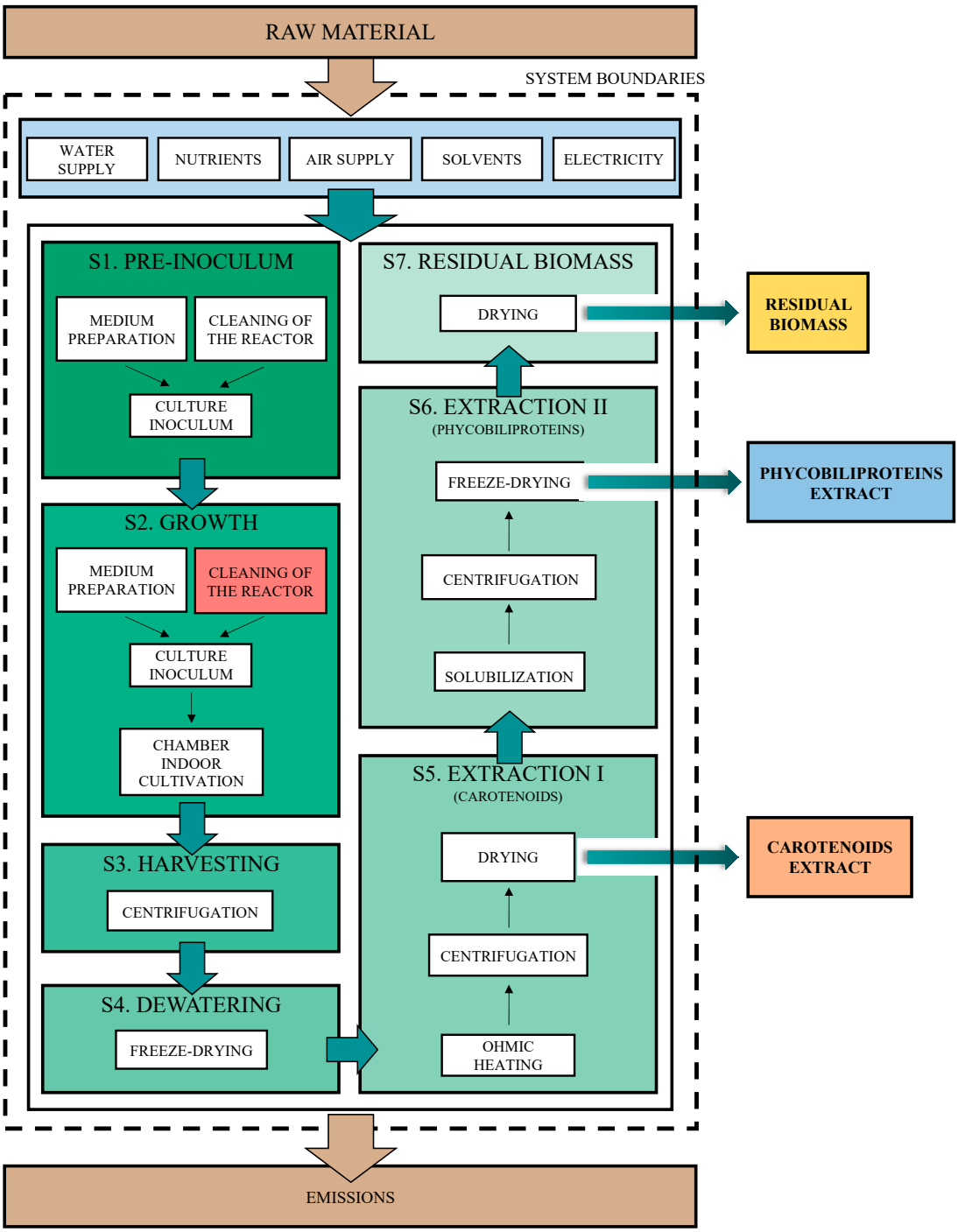

Figure 1. *Cyanobium* sp. bioprocess description and system boundaries evaluated by applying the LCA methodology. The red square indicates significant changes in the process in the scale-up, as S2 "cleaning of the reactor" is not needed in demonstration- and industrial-scale, as it is performed only in S1.

2.3. Process Scale-Up Proposal

Three processes were considered depending on the production volume and reactor type. The first was the available results of the laboratory scale optimisation process (20 L), grown in an indoor growth chamber [15,16] and carried out at CIIMAR, Porto (Portugal); the second was at a demonstration scale, in a flat-panel reactor (140 L), based on the system proposed for *Arthrospira platensis* by Nwoba et al. [35]; and the third scenario is an

industrial-scale open-air tubular bioreactor (100 m^3) proposed by Pereira et al. [36] for *Tetraselmis* sp. (microalgae) and Lopes et al. [17] for *Synechocystis* sp. All scenarios were proposed to take place in Portugal, considering the Portuguese electricity mix for the global inventory, together with the geographic conditions for the outdoor cultures (the annual solar emission and the temperature). Moreover, in all three scenarios, the impact included the production of different inputs to the system.

2.3.1. Laboratory-Scale Process

The laboratory-scale process was based on the proposed optimized process of *Cyanobium* sp. [15,16]. The cultivation was performed in a 20 L polycarbonate cylindrical reactor, the pre-inoculum being performed in a 2 L borosilicate round flask. Both reactors were placed in an Aralab 600 S culture chamber with controlled parameters for light, aeration and temperature.

2.3.2. Demonstration-Scale

The demonstration-scale was based on the process scheme proposed by Nwoba et al. [35] for *Arthrospira platensis* in a single glazed flat-plate photobioreactor (140 L; 1.26 × 1.25 m). The pre-inoculum was performed in the same panel of cultivation, with a reduced volume. With this approach, the need for a second cleaning stage for the culture is avoided, leading to a reduction in water consumption.

To reduce light consumption, the reactor was placed outdoors, and the two-phase light system was secured by covering the reactor with a red filter (LEE 026 bright red). The average annual solar emission and the temperature of Porto (Portugal) (15 °C, 9 h of sunshine; IPMA, 2022 [37]) were considered to evaluate the natural solar energy. Processing parameters included a heater for temperature control, continuous air supply and artificial light, operated 7 h per day to compensate for the reduced photoperiod. Heating sterilisation requirements were estimated using the SuperPro Designer® simulation tool.

The downstream process (S4–7) was performed as described for the laboratory scale, as the amount to be processed is within the operational limits for this equipment. Due to the lack of data for demonstration-scale with *Cyanobium* sp., it has been assumed that the process scale-up will not decrease biomass productivity and extraction yield (as the baseline study has a similar volumetric productivity).

2.3.3. Industrial-Scale (100 m^3)

The industrial-scale was based on the production process proposed by Pereira et al. [36] for *Tetraselmis* sp. (microalgae) and Lopes et al. [17] for *Synechocystis* sp. in a scale-up from laboratory-scale to an outdoor tubular photobioreactor (100 m^3) for the cultivation subsystem (polymethyl methacrylate (PMMA) tubes; $\emptyset_{internal}$ = 56 mm; 96.0 × 4.0 m). Processing parameters for the pre-inoculation and culture subsystems included the annual average solar emission and temperature of Porto, artificial lighting (40 W m^{-2}) for 7 h and average consumption for mixing, aeration and temperature control in large-scale systems (17 MWh day^{-1}) [17,38]. Ozone sterilisation was considered for reactor cleaning and culture medium sterilisation. The downstream process (S3–7) followed the same configuration as in the previous scenarios and the large-scale energy consumption was simulated in the SuperPro Design® 11 tool.

2.4. Inventory Analysis

Comprehensive data on the cyanobacterium cultivation, harvesting and extraction, as well as the amount of chemicals (nutrients, solvents), water supply and waste volume, and electricity consumption, were obtained through laboratory-scale experiments [15,16]. Tables 1–3 include the Life Cycle Inventory considered for the laboratory-scale, demonstration-scale and industrial-scale processes. The electricity values considered for the inventory were based on the Portuguese mix (57% renewable) [39].

Table 1. Global inventory for the laboratory-scale production of *Cyanobium* sp. (20 L) (functional unit: 1 batch).

Inputs from Technosphere					
Materials			*Energy*		
S1. Pre-inoculum			**S1. Pre-inoculum**		
Cleaning of the material			***Medium preparation***		
NaClO	10	g	Autoclave	12	kWh
Tap water	400	mL	***Inoculum***		
Water (deionised)	200	mL	Incubator	70	kWh
Medium preparation			White LED	7.7	kWh
BG11 solids [a]	31.7	g	Air pump	8.4	kWh
Water (deionised)	2	L	**S2. Cultivation**		
S2. Cultivation			***Medium preparation***		
Cleaning of the material			Autoclave	12	kWh
NaClO	100	g	***Inoculum***		
Tap water	4	L	Incubator	218.4	kWh
Water (deionised)	2	L	White LED	22.4	kWh
Medium preparation			Red LED	9	kWh
BG11 solids [a]	285.3	g	Air pump	8.4	kWh
Water (deionised)	18	L	**S3. Harvesting**		
S5. Extraction I			Centrifuge	0.8	
Ethanol	400	mL	**S4. Dewatering**		
Water (deionised)	4	mL	Freezer	1.9	kWh
NaCl	0.1	g	Freeze-drier	48	kWh
S6. Extraction II			**S5. Extraction I**		
Water (deionised)	400	mL	Ohmic Heating	0.1	kWh
			Centrifuge	0.4	kWh
			Rotavapor	1.1	kWh
			S6. Extraction II		
			Agitator	2	Wh
			Centrifuge	0.4	kWh
			Freezer	1.9	kWh
			Freeze-drier	48	kWh
			S7. Residual biomass		
			Dry oven	16.8	kWh
Outputs to technosphere					
Carotenoids' extract	10.8	g			
Phycobiliproteins' extract	10	g			
Residual biomass	19.2	g			
Outputs to environment					
Wastewater	27	L			

[a] Description of BG11 culture medium solids can be found in Pagels et al. [16.]

Table 2. Global inventory for the demonstration-scale production of *Cyanobium* sp. (140 L) (functional unit: 1 batch).

Inputs from Technosphere					
Materials			*Energy*		
S1. Pre-inoculum			**S1. Pre-inoculum**		
Cleaning of the material			***Medium preparation***		
NaClO	7	kg	Heat from steam	36.6	MJ
Tap water	280	L	***Inoculum***		

Water (deionised)	140	L	Heater	5.0	kWh
Medium preparation			Illumination	0.3	kWh
BG11 solids [a]	221.9	g	Air pump	0.8	kWh
Water (deionised)	14	L	**S2. Cultivation**		
S2. Cultivation			***Medium preparation***		
Medium preparation			Heat from steam	329.1	MJ
BG11 solids [a]	2	kg	***Inoculum***		
Water (deionised)	126	L	Heater	100.8	kWh
S5. Extraction I			Illumination	6.2	kWh
Ethanol	2.8	L	Air pump	16.8	kWh
Water (deionised)	28	mL	**S3. Harvesting**		
NaCl	0.6	g	Centrifuge	0.6	kWh
S6. Extraction II			Ultracentrifuge	0.8	kWh
Water (deionised)	2.8	L	**S4. Dewatering**		
			Freezer	1.9	kWh
			Freeze-drier	48	kWh
			S5. Extraction I		
			Ohmic Heating	0.8	kWh
			Centrifuge	0.8	kWh
			Rotavapor	20.4	kWh
			S6. Extraction II		
			Agitator	2	Wh
			Centrifuge	0.8	kWh
			Freezer	1.9	kWh
			Freeze-drier	48	kWh
			S7. Residual biomass		
			Dry oven	16.8	kWh
Inputs from nature					
S1. Pre-inoculum					
Cooling water	1.74	m^3			
S2. Cultivation					
Cooling water	15.7	m^3			
Outputs to technosphere					
Carotenoids' extract	75.6	g			
Phycobiliproteins' extract	70.0	g			
Residual biomass	134.4	g			
Outputs to environment					
Wastewater	565.6	L			

[a] Description of BG11 culture medium solids can be found in Pagels et al. [16].

Table 3. Global inventory for the industrial-scale production of *Cyanobium* sp. (100 m^3) (functional unit: 1 batch).

Inputs from Technosphere					
Materials			*Energy*		
S1. Pre-inoculum			**S1. Pre-inoculum**		
Cleaning of the material			***Cleaning of the material***		
Ozone	1	kg	Ozone sterilisation	12	kWh
Tap water	100	m^3	***Medium preparation***		
Medium preparation			Ozone sterilisation	2.7	kWh
BG11 solids [a]	158.6	kg	***Inoculum***		
Water (deionised)	10	m^3	Growth control [b]	11.9	MWh

Ozone	0.1	kg	Illumination	0.1	MWh
S2. Cultivation			**S2. Cultivation**		
Medium preparation			***Medium preparation***		
BG11 solids [a]	1247	kg	Ozone sterilisation	24.3	kWh
Water (deionised)	90	m^3	***Inoculum***		
S5. Extraction I			Growth control [b]	238	MWh
Ethanol	2	m^3	Illumination	1.5	MWh
Water (deionised)	20	L	**S3. Harvesting**		
NaCl	0.4	kg	Centrifuge	5.4	MWh
S6. Extraction II			**S4. Dewatering**		
Water (deionised)	2	m^3	Freeze-drier	1.9	MWh
			S5. Extraction I		
			Agitator	2	kWh
			Ohmic Heating	67	kWh
			Centrifuge	132	kWh
			Rotary dryer [c]	5.5	GJ
			S6. Extraction II		
			Agitator	2	kWh
			Centrifuge	128	kWh
			Freeze-drier	2	MWh
			S7. Residual biomass		
			Tray dryer [c]	4.1	GJ
Outputs to technosphere					
Carotenoids' extract	54	kg			
Phycobiliproteins' extract	50	kg			
Residual biomass	96	kg			
Outputs to environment					
Wastewater	202	m^3			

[a] Description of BG11 culture medium solids can be found in Pagels et al. [16] [b] Average value for mixing, aeration, and temperature control [17,38] [c] Rotary dryer uses heat from steam.

For the impact assessment, SimaPro 7.3 was used, and the Ecoinvent® database version 3.5 [40] was employed as a secondary data source. For the selection of characterisation factors required to estimate the environmental loads, the ReCiPe 2016 hierarchist Midpoint approach V1.03 World (2010) [41] was used, and a set of impact categories at the midpoint level was selected to report the environmental profiles. The following impacts have been considered: global warming (GW), stratospheric ozone depletion (SOD), terrestrial acidification (TA), freshwater eutrophication (FE), marine eutrophication (ME), terrestrial ecotoxicity (TET), freshwater ecotoxicity (FET), marine ecotoxicity (MET) human carcinogenic toxicity (HCT), human non-carcinogenic toxicity (HNCT), and fossil resource scarcity (FRS).

2.5. Mass and Economic Allocation

As two primary products are obtained (carotenoids and phycobiliproteins extracts), mass and economic allocation were applied to report the environmental loads of each product and determine which has the greatest impact. Mass allocation was based on the amount produced of carotenoid extracts (0.54 g L^{-1} culture) and phycobiliproteins extracts (0.50 g L^{-1} culture). Economic allocation was based on literature data [42]: 3.30 € mg^{-1} for carotenoids (β-carotene as reference) and 14.90 € mg^{-1} for phycobiliproteins (allophycocyanin as reference).

2.6. Sensitivity Analysis

Considering the main critical points identified in the life cycle of the process, i.e., the stages and/or materials that contribute most to the environmental loads, a sensitivity analysis based on alternative scenarios was carried out for each proposed scale, with the aim of improving the proposed process in terms of environmental impact.

3. Results and Discussion

3.1. Laboratory-Scale Profile (20 L)

The characterisation results of the *Cyanobium* sp. bioprocess at the laboratory-scale are shown in Table 4, for the batch, and one litre of culture, with the respective mass and economic allocation for each extract. The relative contribution of each subsystem is represented in Figure 2.

Table 4. Impact assessment results associated with the laboratory-scale process (20 L) per batch and per litre of culture, and the respective allocation for each co-product (mass allocation (MA) and economic allocation (EA)).

Impact [1]	Unit	Per Batch	Per Litre of Culture	Carotenoids		Phycobiliproteins	
				MA	EA	MA	EA
GW	kg $CO_{2\,eq}$	197.3	9.86	5.12	1.79	4.74	8.08
SOD	kg CFC11 $_{eq}$	7.67×10^{-5}	3.84×10^{-6}	1.99×10^{-6}	6.96×10^{-7}	1.84×10^{-6}	3.14×10^{-6}
TA	kg $SO_{2\,eq}$	1.04	5.20×10^{-2}	2.70×10^{-2}	9.43×10^{-3}	2.50×10^{-2}	4.26×10^{-2}
FE	kg P $_{eq}$	7.01×10^{-2}	3.50×10^{-3}	1.82×10^{-3}	6.35×10^{-4}	1.68×10^{-3}	2.87×10^{-3}
ME	kg N $_{eq}$	4.65×10^{-3}	2.32×10^{-4}	1.21×10^{-4}	4.21×10^{-5}	1.12×10^{-4}	1.90×10^{-4}
TET	kg 1.4-DCB	128.1	6.40	3.33	1.16	3.08	5.24
FET	kg 1.4-DCB	2.11	10.57×10^{-2}	5.49×10^{-2}	1.92×10^{-2}	5.08×10^{-2}	8.65×10^{-2}
MET	kg 1.4-DCB	2.99	14.96×10^{-2}	7.77×10^{-2}	2.71×10^{-2}	7.19×10^{-2}	12.25×10^{-2}
HCT	kg 1.4-DCB	4.50	0.22	0.12	0.04	0.11	0.18
HNCT	kg 1.4-DCB	118.5	5.93	3.08	1.07	2.85	4.85
FRS	kg oil $_{eq}$	54.53	2.73	1.42	0.49	1.31	2.23

[1] Impact category: global warming (GW), stratospheric ozone depletion (SOD), terrestrial acidification (TA), freshwater eutrophication (FE), marine eutrophication (ME), terrestrial ecotoxicity (TET), freshwater ecotoxicity (FET), marine ecotoxicity (MET), human carcinogenic toxicity (HCT), human non-carcinogenic toxicity (HNCT), fossil resource scarcity (FRS).

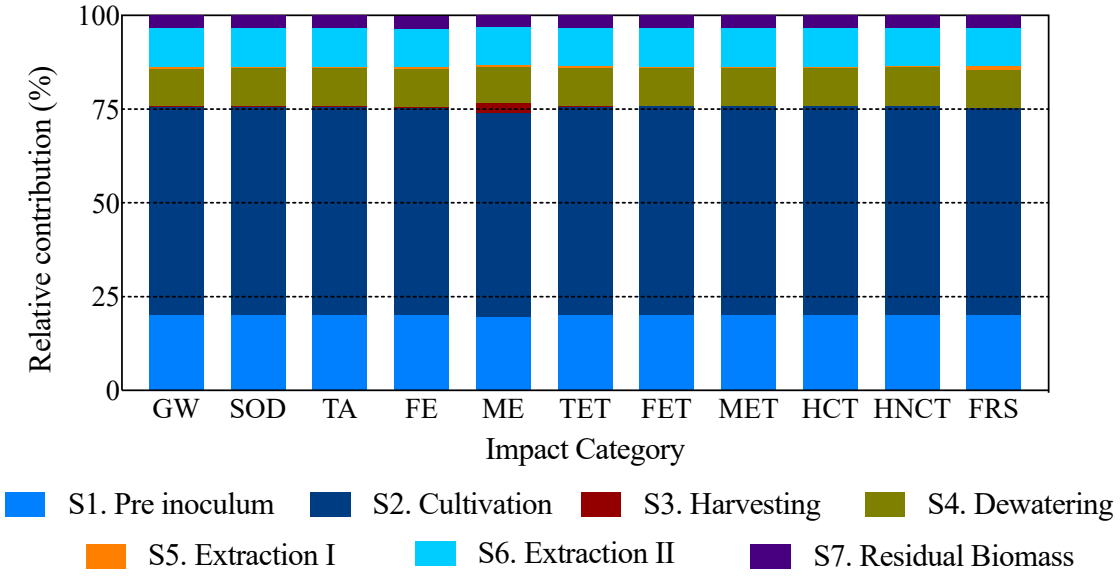

Figure 2. Relative contribution (in %) per subsystem of the laboratory-scale process (20 L) to each impact category: global warming (GW), stratospheric ozone depletion (SOD), terrestrial acidification (TA), freshwater eutrophication (FE), marine eutrophication (ME), terrestrial ecotoxicity (TET), freshwater ecotoxicity (FET), marine ecotoxicity (MET), human carcinogenic toxicity (HCT), human non-carcinogenic toxicity (HNCT), fossil resource scarcity (FRS).

As shown in Figure 2, the relative contribution is relatively constant through all the evaluated impacts: S1 (20%), S2 (55%), S3 (0–3%), S4 (10%), S5 (0–1%), S6 (10%), and S7 (3%), with the cultivation phase (S2) having the most impact. With a more detailed analysis of S2, the impact was found to be mainly related to electricity consumption (about 96%), followed by wastewater (3%) and chemicals (1%).

In terms of mass and economic allocation (Table 4), as both extracts are produced in similar quantities, the allocated impact in mass is similar for both, while as phycobiliproteins are more valuable in the market, the economic allocation implies a higher impact to this extract than to carotenoids.

Previous reports assessing the environmental profiles of cyanobacterial bioprocessing have found the same contribution profile between stages, with cultivation being one of the most environmentally burdensome. Ye et al. [31] observed that *Arthrospira platensis* cultivation accounted for 80% to 95% of the calculated impact for the process, depending on the impact indicator (82% for GW); from that, electricity and sodium bicarbonate were the two main inputs for high impact. In another study, Rodriguez et al. [29] observed that the culture subsystem of the *Arthrospira maxima* bioprocess accounts for up to 56% of the FRS impact. The same effect has also been observed for microalgae, which are the closest competitors to cyanobacteria in the pigment market. Pérez-López et al. [43] assessed the environmental impact of astaxanthin (carotenoid) production by *Haematococcus pluvialis*, and the LCA indicated that for GW, cultivation electricity accounts for 61% of the total impact, suggesting the replacement of the annular photobioreactor with artificial lights to a flat-panel, although still with artificial lighting. To reduce the impact of electricity use, the design of the photobioreactor is critical to achieving energy efficiency, along with the use of artificial lighting. Smetana et al. [30] showed that for *Arthrospira platensis*, replacing an open raceway reactor with a tubular design decreases half of the system impact. On the other hand, the use of outdoor cultivation, which can reduce the impact caused by artificial lighting, could reduce productivity due to fluctuations in light availability [44].

With the analysis of each process subsystem, the main critical points could be identified. The energy demand of the freeze-drying process is expected to be responsible for the largest environmental load. In order to decrease the impact, improvements in the use of wet biomass and/or the modification of the drying procedure is needed. Interestingly, the ohmic heating cell disruption method, used during S5, accounts for less than 1% of the total calculated impact. The efficiency of an electric-based extraction is mainly due to the application of an electric current during a short extraction period (5 min), with rapid and homogeneous heating, which is why this method has less of an impact than others, such as homogenisation or supercritical CO_2 [45]. Furthermore, Käferböck et al. [33] reported that the use of electric fields decreased the environmental burden of phycocyanin extraction by 57–65% when compared to solvent-only extraction. In addition, the drying of residual biomass represents 3% of the total calculated impact and could be overcome for the use of wet pulp for further processing.

Taking into account the main critical points identified, a sensitivity analysis has been carried out based on four alternative scenarios for the lab-scale process: (L1) the use of 20% less electricity; (L2) the use of the European electricity mix instead of Portugal; (L3) the use of the Swedish electricity mix (considered the cleanest in the European Union); and (L4) reduction of the biomass drying step by sending the residue to waste management (19.2 g batch^{-1}). The results of the sensitivity analysis are shown in Figure 3.

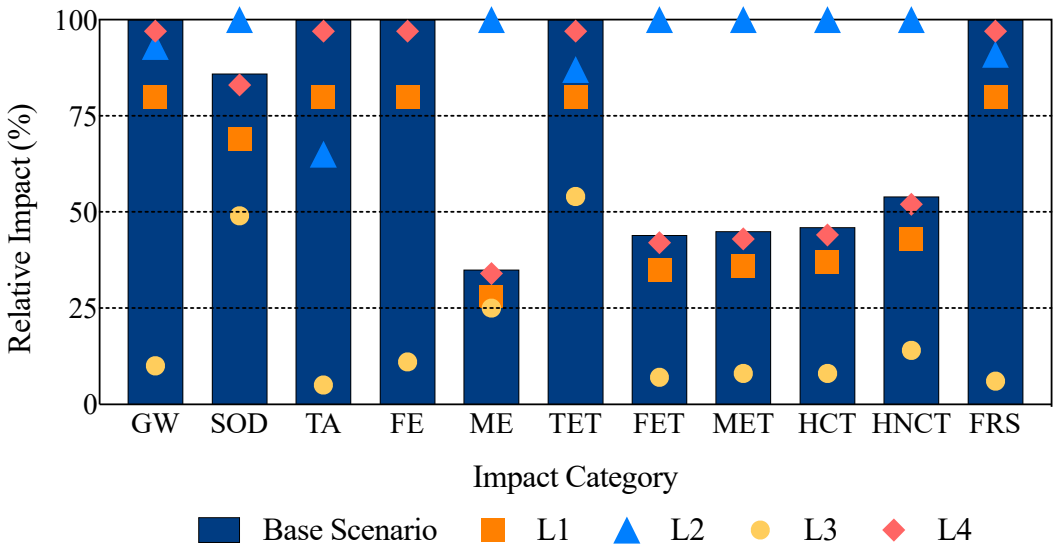

Figure 3. Sensitivity analysis from the laboratory-scale process, considering four alternative scenarios: (L1) the use of 20% less electricity; (L2) the use of Europe electricity mix instead of Portugal; (L3) the use of Swedish electricity mix; (L4) reducing the biomass drying step by sending the residue to waste management. Impact categories: global warming (GW), stratospheric ozone depletion (SOD), terrestrial acidification (TA), freshwater eutrophication (FE), marine eutrophication (ME), terrestrial ecotoxicity (TET), freshwater ecotoxicity (FET), marine ecotoxicity (MET), human carcinogenic toxicity (HCT), human non-carcinogenic toxicity (HNCT), fossil resource scarcity (FRS).

By reducing the electricity consumption by 20% (scenario L1), the environmental load of the process was also reduced by 20%, which corroborates the critical point found in the baseline scenario, where electricity corresponds to most of the total impact. Moreover, the substitution of the Portuguese electricity mix with that of Europe (Scenario L2) leads to a reduction in the categories GW, TA, TET and FRS, while in the others some increases could be seen, highlighting that the impact of FE, ME, FET, MET and HCT increased by more than 100%, probably due to the lower share of renewable energy in Europe (22.1%) compared to Portugal (33.9%) [46]. In the third scenario (L3, Swedish energy emissions use), all factors decreased, on average by 74%. Sweden is considered the European country with the highest rate of renewable electricity (60.1%), mainly due to the use of hydropower (39.3%) [47], while in Portugal, although hydropower and wind account for a large share of energy consumption, coal and natural gas can increase the overall impact of this electricity mix [39].

Finally, by eliminating the residual biomass drying subsystem (scenario L4), and instead of using this waste as a by-product, sending it to waste management, the environmental burden of the process would decrease by 3%. However, due to the lack of studies on the use of this specific biomass as fertiliser, it is not possible to assume whether this reduction is a real advantage or a disadvantage. Considering the nitrogen content of the residue of *Cyanobium* sp. spent biomass (12.1%) and reports about other species, such as *Arthrospira platensis*, it could be assumed that a subproduct is more advantageous than sending this biomass to waste management [48].

3.2. Demonstration-Scale (140 L)

Regarding the environmental assessment at the demonstration scale, the characterisation results of the *Cyanobium* sp. bioprocess are shown in Table 5 for the batch, and one litre of culture, with the respective mass and economic allocation for each extract. The relative contribution of each subsystem is depicted in Figure 4.

Table 5. Impact assessment results associated with the demonstration-scale process (140 L) per batch and per litre of culture, and the respective allocation for each co-product (mass allocation (MA) and economic allocation (EA)).

Impact [1]	Unit	Per Batch	Per Litre of Culture	Carotenoids		Phycobiliproteins	
				MA	EA	MA	EA
GW	kg $CO_{2\ eq}$	165.26	1.18	0.61	0.21	0.57	0.97
SOD	kg CFC11 $_{eq}$	7.18×10^{-5}	5.13×10^{-7}	2.66×10^{-7}	9.29×10^{-8}	2.46×10^{-7}	4.20×10^{-7}
TA	kg $SO_{2\ eq}$	7.25×10^{-1}	5.18×10^{-3}	2.69×10^{-3}	9.39×10^{-4}	2.49×10^{-3}	4.24×10^{-3}
FE	kg P $_{eq}$	5.41×10^{-2}	3.86×10^{-4}	2.00×10^{-4}	7.00×10^{-5}	1.86×10^{-4}	3.16×10^{-4}
ME	kg N $_{eq}$	7.19×10^{-3}	5.14×10^{-5}	2.67×10^{-5}	9.32×10^{-6}	2.47×10^{-5}	4.21×10^{-5}
TET	kg 1.4-DCB	151.75	1.08	0.56	0.20	0.52	0.89
FET	kg 1.4-DCB	1.58	1.13×10^{-2}	5.86×10^{-3}	2.04×10^{-3}	5.42×10^{-3}	9.23×10^{-3}
MET	kg 1.4-DCB	2.25	1.61×10^{-2}	8.36×10^{-3}	2.92×10^{-3}	7.74×10^{-3}	1.31×10^{-2}
HCT	kg 1.4-DCB	3.37	2.41×10^{-2}	1.25×10^{-2}	4.37×10^{-3}	1.16×10^{-2}	1.97×10^{-2}
HNCT	kg 1.4-DCB	85.81	0.61	0.32	0.11	0.29	0.50
FRS	kg oil $_{eq}$	49.37	0.35	0.18	0.06	0.17	0.29

[1] Impact category: global warming (GW), stratospheric ozone depletion (SOD), terrestrial acidification (TA), freshwater eutrophication (FE), marine eutrophication (ME), terrestrial ecotoxicity (TET), freshwater ecotoxicity (FET), marine ecotoxicity (MET), human carcinogenic toxicity (HCT), human non-carcinogenic toxicity (HNCT), fossil resource scarcity (FRS).

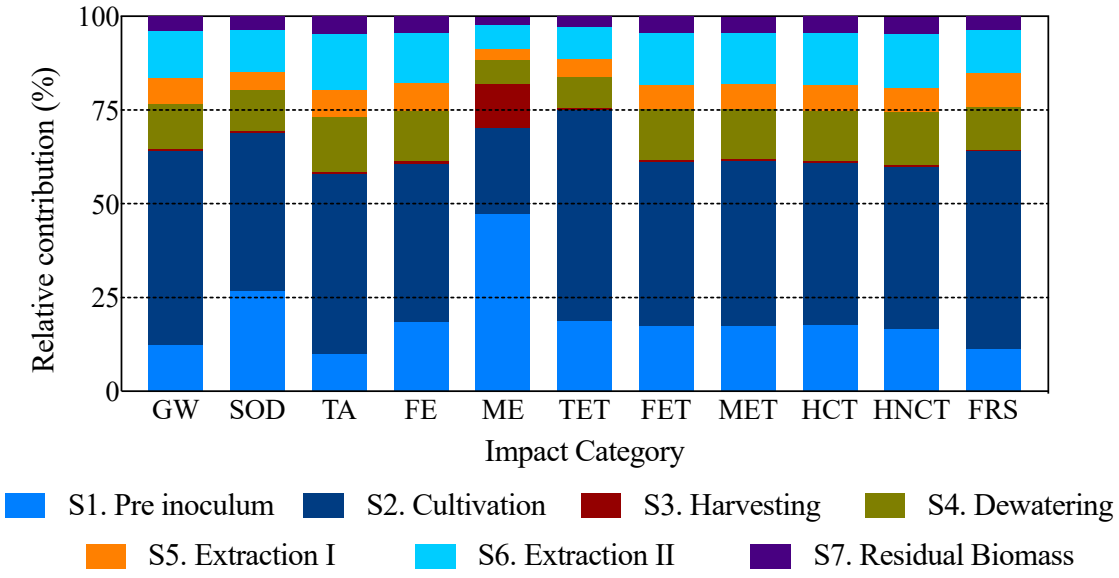

Figure 4. Relative contribution (in %) per subsystem of the demonstration-scale process (140 L) to each impact category: global warming (GW), stratospheric ozone depletion (SOD), terrestrial acidification (TA), freshwater eutrophication (FE), marine eutrophication (ME), terrestrial ecotoxicity (TET), freshwater ecotoxicity (FET), marine ecotoxicity (MET), human carcinogenic toxicity (HCT), human non-carcinogenic toxicity (HNCT), fossil resource scarcity (FRS).

As shown in Figure 4, the relative contribution was again relatively constant in almost all impact categories assessed, with the average contribution of S1 (11–17%), S2 (42–56%), S3 (0–1%), S4 (13%), S5 (5–7%), S6 (11–13%) and S7 (3–5%), with the cultivation phase (S2) having the greatest impact. The exception was observed in the ME impact category, where pre-inoculum accounts for 47% of the total impact, while cultivation only represents 23%, which can be explained by a more detailed analysis of the sub-systems. The relative contribution of S1 showed that the impact is mainly due to the reactor cleaning stage (ca. 70–80%), as it requires the use of significant amounts of sodium hypochlorite (responsible for 99% of this impact) and leads to the production of a large amount of

wastewater which must be sent for further treatment. In addition, it should be noted that electricity and heat (for sterilisation) are still responsible for a large part of the impacts in the cultivation subsystem (S2): approximately 70% for electricity and 20% for thermal sterilisation. Furthermore, the environmental profiles of each subsystem appeared again as a considerable hotspot for the energy consumption for the drying processes (freeze-drying in dewatering and extraction II and rotavapor in extraction I).

In contrast to the laboratory scale environmental results, the total batch impact (Table 5) showed a reduction in 9 of the 11 impact categories assessed, a result even more pronounced for the impact of 1 litre of culture broth, where the demonstration scale showed a drastic reduction in impact. The reason for this is mainly due to the change in the photobioreactor. A growth chamber with artificial lighting, ideal for optimisation processes, consumes a large amount of energy; in contrast, an open-air system consumes less than half of the energy, even though it is heated all day and illuminated during the night. On the other hand, scale-up processes can generally lead to losses in biomass productivity [49]; here, one of the main assumptions was that productivity would be the same, as the reactor showed similar volumetric productivity in the experimental study with *Arthrospira platensis* [35]. However, it is important to note that the use of different species can have a large impact on the efficiency of the system: approximately 20% for a demonstration scale and up to 50% for a large production scale [49].

Regarding sterilisation procedures, identified as an expressive hotspot for the demonstration scale, ozone sterilisation has been proposed as a viable alternative to heat sterilisation in large-scale microalgae production [8], which could reduce the high impact caused by this step. Furthermore, Pérez-López et al. [43] proposed the same ozone sterilisation for the reactor cleaning stage, although no differences were found in the environmental profile, when compared to the reverse osmosis and UV filtration for the sterilisation of the culture medium.

For a better interpretation and proposal of a more efficient process, a sensitivity analysis was performed based on four different scenarios for the demonstration scale process, taking into account the identified critical points of the life cycle of the process: (D1) the use of ozone sterilisation for cleaning (0.12 kWh m^{-3} + 10 g m^{-3}; Pérez-López et al. [43]); (D2) the use of ozone sterilisation of the culture medium (0.27 kWh m^{-3} + 10 g m^{-3} ozone; Acién et al. [8]); (D3) the use of reverse osmosis (7.71 kWh m^{-3}) and UV sterilisation (0.35 kWh m^{-3}) of the culture medium (Pérez-López et al. [43]); (D4) reducing the biomass productivity by 20 %. The results of the sensitivity analysis are shown in Figure 5.

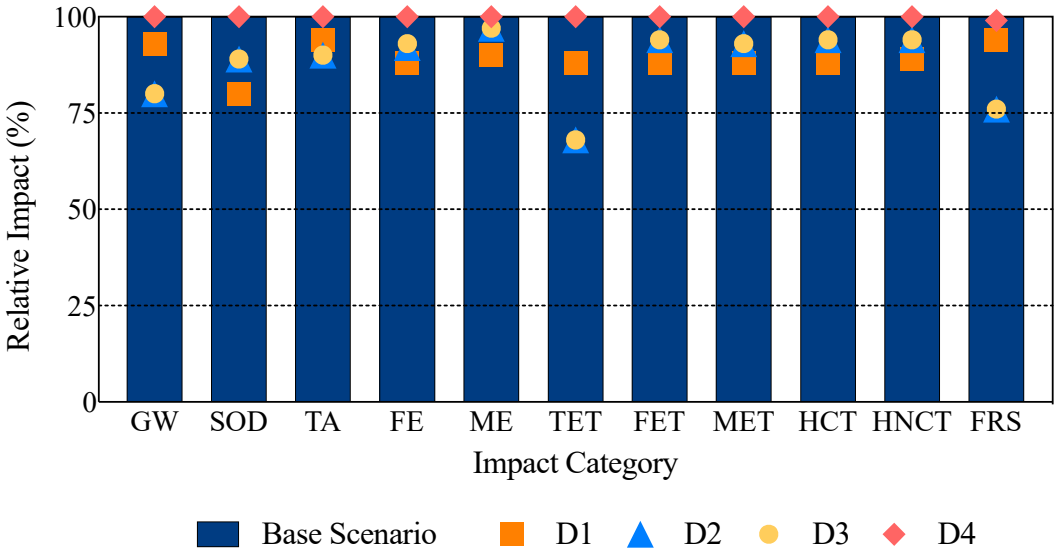

Figure 5. Sensitivity analysis from the demonstration-scale process, considering four alternative scenarios: (D1) the use of ozone sterilisation for cleaning; (D2) the use of ozone sterilisation of the

culture medium; (D3) the use of reverse osmosis and UV sterilisation of the culture medium; (D4) reducing the biomass productivity by 20%. Impact categories: global warming (GW), stratospheric ozone depletion (SOD), terrestrial acidification (TA), freshwater eutrophication (FE), marine eutrophication (ME), terrestrial ecotoxicity (TET), freshwater ecotoxicity (FET), marine ecotoxicity (MET), human carcinogenic toxicity (HCT), human non-carcinogenic toxicity (HNCT), fossil resource scarcity (FRS).

The use of ozone sterilisation for reactor cleaning (Scenario D1) reduces environmental loads by approximately 10% of all impact categories assessed, except for SOD, where the reduction was 20%. This scenario was proposed by Pérez-López et al. [43], although the authors did not observe significant differences between ozone sterilisation and chemical sterilisation, contrary to the *Cyanobium* bioprocess. Moreover, the authors proposed the use of ozone sterilisation for large-scale production due to the higher feasibility of this method compared to chemical sterilisation.

The use of ozone (Scenario D2) or reverse osmosis and UV filtration (Scenario 3) for medium sterilisation showed a similar impact between the two, and large impact reductions compared to the baseline scenario (heat sterilisation), mainly in GW (20%), TET (30%) and FRS (25%). Between the two, Scenario D2 would be more affordable, as ozone would also be used for reactor cleaning, and reverse osmosis and UV filtration require a two-step process, which is more labour-intensive and time-consuming.

Finally, the reduction of biomass productivity (Scenario D4) did not show any significant change in the environmental profile considering the batch, as the only change in inputs relates to solvent volumes S5 and S6, and ohmic heating energy consumption S5.

3.3. Industrial-Scale (100 m³)

The evaluation of the environmental impact from the industrial-scale reactor (100 m^3) was characterized in terms of the total impact of the batch, and one litre of culture, with the respective mass and economic allocation for each extract (Table 6). The relative contribution of each subsystem of the system is depicted in Figure 6.

Table 6. Impact assessment results associated with the industrial-scale process (100 m^3) per batch and per litre of culture, and the respective allocation for each co-product (mass allocation (MA) and economic allocation (EA)).

Impact [1]	Unit	Per Batch	Per Litre of Culture	Carotenoids		Phycobiliproteins	
				MA	EA	MA	EA
GW	kg $CO_{2\,eq}$	105.16×10^3	1.05	0.55	0.19	0.51	0.86
SOD	kg CFC11 $_{eq}$	4.16×10^{-2}	4.16×10^{-7}	2.16×10^{-7}	7.55×10^{-8}	2.00×10^{-7}	3.41×10^{-7}
TA	kg $SO_{2\,eq}$	545.05	5.45×10^{-3}	2.83×10^{-3}	9.88×10^{-4}	2.62×10^{-3}	4.46×10^{-3}
FE	kg P $_{eq}$	37.61	3.76×10^{-4}	1.95×10^{-4}	6.82×10^{-5}	1.81×10^{-4}	3.08×10^{-4}
ME	kg N $_{eq}$	3.74	3.75×10^{-5}	1.95×10^{-5}	6.80×10^{-6}	1.80×10^{-5}	3.07×10^{-5}
TET	kg 1.4-DCB	74.47×10^3	0.74	0.39	0.14	0.36	0.61
FET	kg 1.4-DCB	1.13×10^3	1.13×10^{-2}	5.89×10^{-3}	2.06×10^{-3}	5.45×10^{-3}	9.28×10^{-3}
MET	kg 1.4-DCB	1.60×10^3	1.60×10^{-2}	8.33×10^{-3}	2.91×10^{-3}	7.71×10^{-3}	1.313×10^{-2}
HCT	kg 1.4-DCB	2.40×10^3	2.40×10^{-2}	1.25×10^{-2}	4.35×10^{-3}	1.15×10^{-2}	1.96×10^{-2}
HNCT	kg 1.4-DCB	62.83×10^3	0.63	0.33	0.11	0.30	0.51
FRS	kg oil $_{eq}$	29.96×10^3	0.30	0.16	0.05	0.14	0.25

[1] Impact category: global warming (GW), stratospheric ozone depletion (SOD), terrestrial acidification (TA), freshwater eutrophication (FE), marine eutrophication (ME), terrestrial ecotoxicity (TET), freshwater ecotoxicity (FET), marine ecotoxicity (MET), human carcinogenic toxicity (HCT), human non-carcinogenic toxicity (HNCT), fossil resource scarcity (FRS).

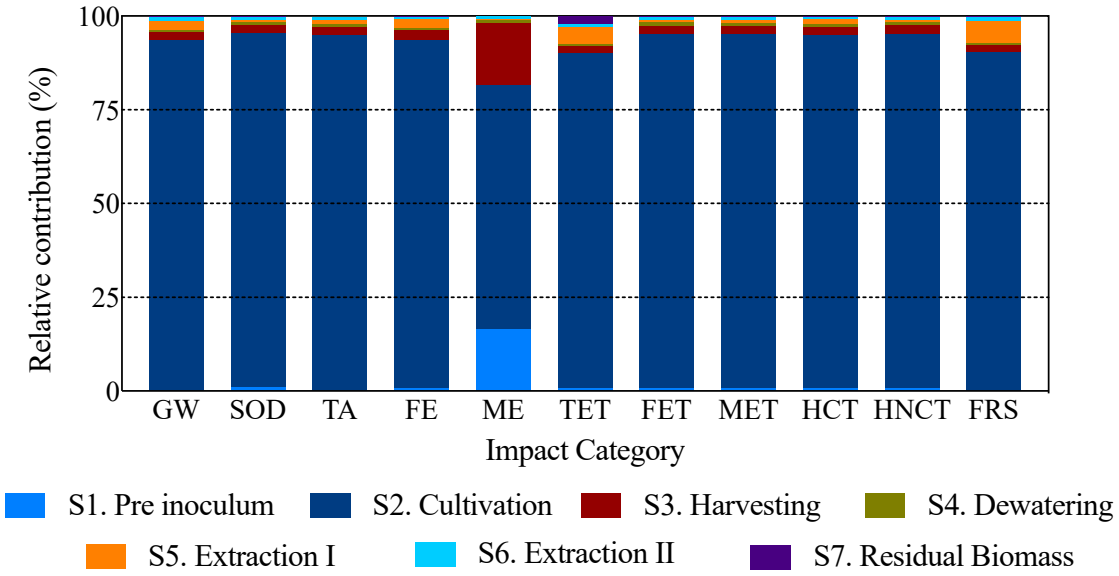

Figure 6. Relative contribution (in %) per subsystem of the industrial-scale process (100 m^3) to each impact category: global warming (GW), stratospheric ozone depletion (SOD), terrestrial acidification (TA), freshwater eutrophication (FE), marine eutrophication (ME), terrestrial ecotoxicity (TET), freshwater ecotoxicity (FET), marine ecotoxicity (MET), human carcinogenic toxicity (HCT), human non-carcinogenic toxicity (HNCT), fossil resource scarcity (FRS).

The relative contribution (Figure 6) was again mostly impacted by the pre-inoculum and cultivation subsystems, with a higher proportion to S2 (95%), for the other subsystems; the average contribution was S1 (0.2–1%), S3 (1%), S4 (0.5%), S5 (0.5–4%), S6 (0.5%) and S7 (0.2%), except for ME, where the cultivation subsystem decreased to 65% and harvesting increased to 16.5% due to the wasted culture medium. Contrarily to the demonstration-scale, the detailed analysis of the subsystems S2 showed that energy was responsible for about 98% of the impact, except for marine eutrophication (ME) in S1, which is highly impacted by the wastewater discharged during the cleaning process, and in S2, which was impacted using sodium nitrate.

However, the energy requirements of large-scale production processes have been assessed in detail previously by Lopes et al. [17] and Perez-Lopez et al. [38], and the culture control equipment (i.e., mixing, aeration, and temperature) depends on the time of year and outside temperature. The assessed values are an average for annual consumption in the Netherlands and Portugal. An alternative to reduce the impact is to reduce emissions from downstream processes, such as the dewatering process.

Drying is one of the most crucial steps in determining the efficiency of the downstream process for cyanobacteria, and the most common drying methods used in large-scale production are freezing, spray and convection drying, and the feasibility of these methods depends on the desired end-use application [50]. Seghiri et al. [51] compared conventional convection (tray-) drying, freeze-drying and spray drying for phycobiliprotein extraction in *Arthrospira platensis*, leading to the selection of tray-drying (70 °C for 8 h) as the optimal method, although freeze-drying resulted in high purity of the final product. Furthermore, Pérez-López et al. [43] proposed freeze-drying for the laboratory scale and spray drying for the pilot scale, as this type of equipment is less time-consuming.

Furthermore, to reduce the impact on the upstream process, the cyanobacteria could be cultured under the semi-continuous mode, using a fraction of the final culture as pre-inoculum for the next one. A different strain of *Cyanobium* sp. has been studied in semi-continuous mode before for up to ten cycles on a laboratory-scale [52]. This could represent a reduction of impact from cleaning the reactor and pre-inoculation (S1), which is only needed once every ten batches. Furthermore, the scale-up processing can lead to a

substantial decrease in biomass productivity, and on a large scale, the loss can be up to 50% of the production [49].

Considering the previous discussion, a sensitivity analysis was carried out based on four different scenarios for the industrial-scale process, taking into consideration the identified hotspots of the downstream process life cycle: (I1) reducing the biomass productivity by 50% [49]; (I2) the use of spray-drying of biomass [38]; (I3) the use of tray-drying of biomass [51]; (I4) and the use of a semi-continuous process (reduction of S1 impact) [52]. The results of the sensitivity analysis are shown in Figure 7.

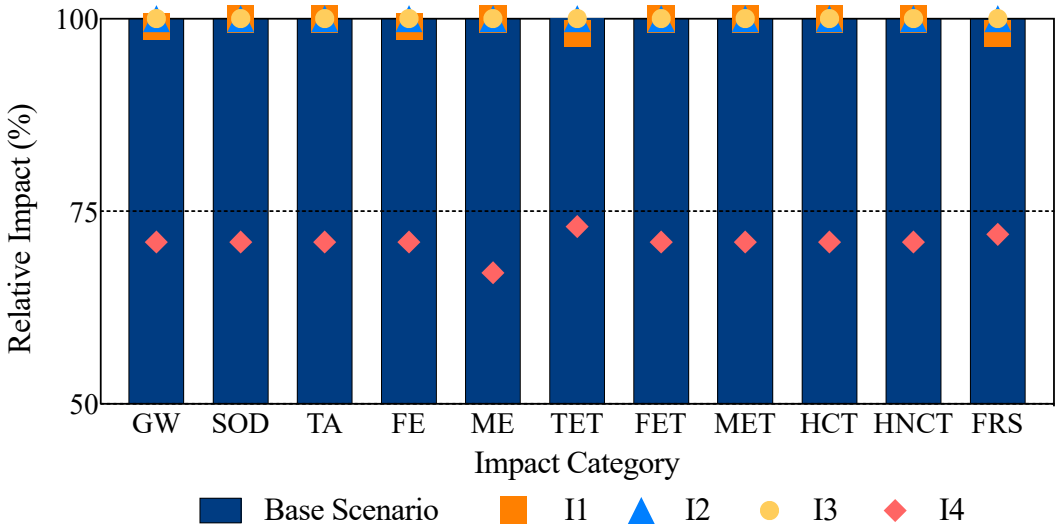

Figure 7. Sensitivity analysis from the industrial-scale process, considering four alternative scenarios: (I1) reducing biomass production by 50%; (I2) the use of spray-drying of biomass; (I3) the use of tray-drying of biomass; (I4) the use of a semi-continuous process (reduction of S1 impact). Impact categories: global warming (GW), stratospheric ozone depletion (SOD), terrestrial acidification (TA), freshwater eutrophication (FE), marine eutrophication (ME), terrestrial ecotoxicity (TET), freshwater ecotoxicity (FET), marine ecotoxicity (MET), human carcinogenic toxicity (HCT), human non-carcinogenic toxicity (HNCT), and fossil resource scarcity (FRS).

If a reduction of 50% in the biomass (Scenario I1) is considered, the environmental load relative to the downstream processing is reduced, as less solvents are used for the extraction, leading to a lower overall impact. On the other hand, the reduction in productivity increases the impact on a unit of product, as the extracts are also reduced by half.

When it comes to alternatives for dewatering, Scenarios I2 and I3 have no significant reduction in the environmental load of the system, although in a detailed analysis of subsystem S4 (Figure 8), it is possible to see that spray-drying and tray-drying have a reduced impact when compared to freeze-dying, except for TET, where steam has more impact than electricity mix; overall, tray-drying is the one with less impacts. Moreover, it is important to evaluate, in terms of experimental data, how the *Cyanobium* sp. biomass would be affected in terms of pigment composition when dried with these different approaches, in order to have a more secure decision.

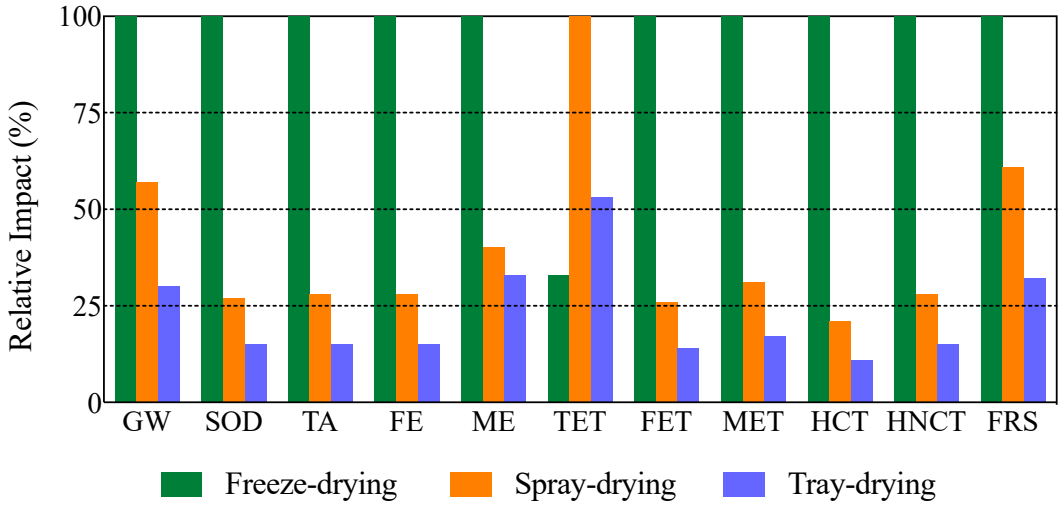

Figure 8. Sensitivity analysis of the dewatering subsystem (S4) on industrial scale (100 m^3) to each impact category: global warming (GW), stratospheric ozone depletion (SOD), terrestrial acidification (TA), freshwater eutrophication (FE), marine eutrophication (ME), terrestrial ecotoxicity (TET), freshwater ecotoxicity (FET), marine ecotoxicity (MET), human carcinogenic toxicity (HCT), human non-carcinogenic toxicity (HNCT), fossil resource scarcity (FRS).

Thus, the semi-continuous cultivation (Scenario I4) reduces the overall impact by 30%, even though it reduces the biomass productivity and consequently the downstream process by 10%. Moreover, the assumption that *Cyanobium* sp. can revert the metabolism from red-phase to white-phase has not been studied, and if compared to the other two-phase cultures, such as the microalgae *Haematococcus pluvialis*, the switch from the stress phase to the growth phase is very optimistic and does not consider that the culture requires a laborious step to revert the stress conditions [53].

After the evaluation and proposal of scale-up processing, and to give an idea of the advantage of strategic thinking of scale-up, the relative impact of each proposed system was compared considering one litre of culture (Tables 4–6), and it is clear how the outdoor culture, with controlled cultivation productions, led to a reduction in all of the impacts from laboratory to demonstration and industrial scales in a range of 80% to 90%. Moreover, the difference between demonstration and industrial scales is less evident, as it increases in some impact categories and decreases in others, which indicates that the scale-up model was successful.

4. Conclusions

The purpose of this study was to identify environmental hotspots in pigment production from *Cyanobium* sp. to provide specific information in the decision-making for sustainable scale-up. The results showed that from laboratory to industrial scale, the largest impact comes from the cultivation of the cyanobacteria, mainly due to temperature control and their electricity consumption, as confirmed by some of the sensitivity analyses performed. In addition, the use of simulation tools has provided fundamental information on downstream processing and the potential impact caused, results that are in the same range of values as those found in the literature. Finally, the scale-up process reduced the impact when allocated to pigment extracts, even if the reduction of biomass productivity in the large-scale process is reduced by 50%. To sum up, this report can guide the strategy of cyanobacterial production with lower environmental impacts by identifying those critical factors that are the main drawbacks to be overcome.

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administration, M.T.M.; funding acquisition, M.T.M. All authors have read and agreed to the published version of the manuscript.

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