Journal of
Experimental and Theoretical Analyses

MDPI

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Sustainability Assessment in Recombinant Human Insulin Production—Evaluating the Environmental Impacts of Microbial Growth Medium Components and Formulations

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Abstract

According to the International Diabetes Federation, approximately 537 million adults suffered from diabetes in 2021, a number that is projected to rise to 783 million by 2045. Insulin is a hormone produced by the pancreas that regulates blood glucose levels; for people suffering from diabetes, insulin activity may be reduced or absent, and therefore, administration of insulin may be necessary to maintain healthy blood glucose levels. Recombinant human insulin is commercially produced using a variety of host microorganisms, such as bacteria and yeast. Nevertheless, few studies have assessed the environmental impacts associated with different upstream medium formulations and their contribution to the overall environmental footprint of recombinant insulin production. Here, Life Cycle Assessment (LCA) is conducted on various upstream media used in insulin production—including pre-cultivation, growth, feed, and induction media—capturing the impacts associated with both their supply chains and their on-site preparation. Hotspots of environmental impacts are identified, and different alternatives for input materials and process conditions are compared in terms of impacts. The findings reported here can serve to guide process and sustainability optimization of the upstream production process from an operational process perspective. Finally, the identification of hotspots enables the implementation of impact reduction measures in bioprocess design, which have the potential to significantly improve the sustainability of insulin production.

Keywords: Life Cycle Assessment (LCA); insulin; biopharmaceuticals; fermentation; microbial growth medium; recombinant protein

Academic Editors: Claudia Letizia Bianchi, Serena Biella and Marco Rossi

Received: 30 June 2025
Revised: 3 September 2025
Accepted: 9 September 2025
Published: 15 September 2025

Citation: Chatzipanagiotou, K.-R.; Pappas, A.; Petrakli, F.; Antonaropoulos, G.; Koumoulos, E.P. Sustainability Assessment in Recombinant Human Insulin Production—Evaluating the Environmental Impacts of Microbial Growth Medium Components and Formulations. *J. Exp. Theor. Anal.* **2025**, *3*, 28. https://doi.org/10.3390/jeta3030028

1. Introduction

Insulin is a peptide hormone, produced and secreted by the pancreas, which functions in the homeostasis of glucose concentration in the plasma and regulates carbohydrate, fat, and protein metabolism [1]. In humans, insulin is a polypeptide of 51 amino acids and has a molecular weight of 5808 Da. It is produced by beta cells of the pancreas and consists of an A-chain (21 amino acids) and a B-chain (30 amino acids), connected to each other via disulfide bonds. Human insulin is produced as a single-chain molecule (proinsulin), wherein the A- and B-chains are linked together via a short polypeptide region called C-peptide. The conversion of proinsulin to native insulin involves the folding of the molecule,

the formation of a disulfide bridge between the A- and B-chains, and proteolytic cleavage, resulting in the release of C-peptide [2–4].

Diabetes mellitus, commonly referred to as diabetes, is a chronic metabolic disease and is associated with disorder in insulin secretion and its effects on metabolism. Two main types of diabetes exist, based on their effect related to insulin. In type I diabetes, the production of insulin is deficient due to impaired function of the beta cells of the pancreas, and therefore, daily administration of insulin is needed. In type II diabetes, resistance is observed in the effects of insulin, or insulin secretion is decreased [1]. According to the World Health Organization [5], in 2022, 14% of adults (aged 18 years and older) suffered from diabetes. According to a 2018 study published by Health Action International [6], the overall number of patients with type II diabetes (i.e., 96.5% of all diabetes patients) is estimated to increase from 405.6 million in 2018 to 510.8 million in 2030. Furthermore, the overall insulin use is projected to increase from 516.1 million to 633.7 million 1000 IU vials per year between 2018 and 2030 [7]. In fact, antidiabetic medications were ranked the second highest group in terms of revenue, after oncologic drugs, based on data from 2018 [8]. Therefore, investigating methods to improve the production of antidiabetic drugs, such as insulin, has the potential to lead to significant impacts in the pharmaceutical sector, both in terms of revenue as well as environmental sustainability.

Since its discovery in 1922, various attempts have been made to treat diabetes using insulin, using injections of pancreatic extract and insulin produced from animal pancreas, as well as to prolong the duration of action (thus decreasing the frequency of injections) of animal-derived insulin. The transition from animal-derived to recombinant insulin took place in 1978, based on the work of Genentech using *Escherichia coli* (*E. coli*) [9]. Thereafter, insulin was the first licensed drug produced using recombinant DNA technology, developed by Genentech and licensed and marketed by Eli Lilly in 1982 using *E. coli* [4], while recombinant insulin production with *Saccharomyces cerevisiae* (*S. cerevisiae*) was initiated in 1987 [10]. Production of recombinant proteins like insulin with microorganisms (MOs) consists of upstream and downstream production steps. Upstream production involves the genetic engineering of host cells to express the insulin gene, followed by cultivation in bioreactors under optimized conditions to maximize biomass and protein expression. Downstream production includes the recovery of insulin from the host cells, typically starting with cell lysis and the isolation of inclusion bodies, followed by solubilization, refolding, enzymatic processing to convert proinsulin to active insulin, and purification using chromatographic techniques [11,12].

Two main approaches have been investigated for the production of human insulin with *E. coli* biocatalyst. One approach involves the cytoplasmic synthesis of proinsulin, containing both the A- and B-chains within its molecule, fused to a fusion protein partner, which can be separated from the proinsulin using cyanogen bromide or proteolytic cleavage during downstream processing. The proinsulin is converted to native insulin via proteolytic cleavage of the connecting C-peptide, thus releasing the A- and B-chains, connected to each other via disulfide bonds. Another approach involves the separate production of the A- and B-chains by different biocatalysts, with each chain linked to fusion protein partners. The fused A- and B-chains are thereafter separately purified, followed by combination of the two chains and formation of the native insulin [3,13–15]. The fusion partner serves to prevent the intracellular degradation of the proinsulin by directing and promoting the formation of inclusion bodies, i.e., insoluble, dense, amorphous aggregates of the produced proteins. The formation of inclusion bodies offers advantages in the production of recombinant proteins with *E. coli*, as they facilitate the isolation of the protein, and they protect the produced protein from cleavage by intracellular proteases [16].

Several advantages have been reported for using *E. coli* as the biocatalyst for recombinant protein production, such as the high growth rate, high yield, higher average specific productivity (compared to yeast), ease of handling, simple medium requirement for its growth, and cost-effective cultivation [4]. However, several disadvantages have also been reported, including loss of plasmids and antibiotic properties, gene expression via unsolicited inducers, intracellular accumulation of heterologous proteins in the form of inclusion bodies, improper protein refolding, lack of, or limited capability of, post- translational modifications (e.g., formation of disulfide bonds), metabolic burden and stress to the host organism due to the produced proteins, endotoxin contamination, poor secretion, proteolytic digestion, and complexity in downstream processing [4].

Insulin production has also been successfully carried out using yeast, for example, *S. cerevisiae*, *Pichia pastoris* (*P. pastoris*), and *Hansenulla polymorpha*, as the biocatalyst. Several advantages have been reported for the use of yeast, as they can grow fast under cultivation, are cost-effective and easy to scale up, present higher maximum biomass concentrations during cultivation (compared to bacteria), and are easy to handle. Eukaryotic organisms such as yeast can furthermore perform the production and proper folding of several mammalian proteins, as well as post-translational modifications, such as phosphorylation, glycosylation, acetylation, and acylation. Proteins produced by *S. cerevisiae* are directly secreted in the reaction medium, which decreases the requirements for product harvesting and purification from biomass [4,10]. One disadvantage reported for *S. cerevisiae* is that it performs high-mannose-type N-glycosylation, forming glycan structures that are immunogenic in humans and are recognized and rapidly removed from circulation via mannose receptors in the human body. Consequently, these glycans have a short serum half-life and thus reduced therapeutic efficacy. To that end, efforts are being made to engineer yeast that can perform human-like N-glycosylation patterns [4,10].

While indispensable to ensure quality of life, pharmaceuticals pose a significant risk to the environment throughout their life cycle. Environmental impacts can be associated with the manufacturing phase of pharmaceuticals, where large volumes of water and solvents as well as significant amounts of energy and resources are consumed, consequently leading to the generation of large quantities of waste requiring treatment [17–19]. Another source of environmental impacts during the production of pharmaceuticals relates to the release of intermediates and products to the local environment, e.g., via accidental release, emissions, and after treatment of waste generated during production. This is particularly concerning when pharmaceutical production takes place in low-income countries, where environmental regulations and measures against the release of pollutants are less strict, leading to significant levels of environmental pollution from pharmaceuticals [19,20]. Additional environmental impacts arise from the packaging and transportation of pharmaceuticals. A significant source of environmental pollution is associated with the use phase, wherein (partially) metabolized pharmaceuticals and residues are excreted by humans into the sewage system. From there, they get (in part) removed from wastewater during treatment, as well as undergo transformation, and eventually end up in the environment, as part of the outflow of wastewater treatment plants. Finally, another source of environmental pollution is the improper disposal of unused or expired pharmaceuticals by consumers (e.g., down the drain or in municipal solid waste) [19,21,22].

As for any manufacturing sector, several environmental policies exist, aiming to regulate and minimize the environmental impacts of the pharmaceutical sector in general, and biopharmaceuticals in particular. For example, an extensive overview of relevant regulations has been reported by Chau (2021) [23]. Nevertheless, the existing regulations in place pose little incentive for biopharmaceutical companies to take measures against the environmental impacts of their manufacturing process, particularly if such measures do not

result in economic benefits. This is partially due to the scale of production and, therefore, emissions. Environmental regulations prioritize industries with high emissions, while biopharmaceutical production typically operates at smaller scales [23], depending, of course, on the exact biopharmaceutical under assessment, as the scales may vary for different compounds. An additional reason why the environmental impacts of biopharmaceuticals may be overlooked is that several substances utilized in their synthesis are not classified as hazardous as part of the Control of Substances Hazardous to Health, within the European Directive on reducing industrial emissions (Directive 2010/75/EU, 2010, [24]) [23]. Finally, the section on organic fine chemicals (which pertains to biopharmaceuticals) in the guideline for Best Available Techniques (within Directive 2010/75/EU [24]) primarily prioritizes emissions of Volatile Organic Compounds, wastewaters containing high concentrations of non-degradable organic compounds, spent solvents, and non-recyclable waste. However, in the case of biopharmaceuticals, these are not particularly relevant emission categories in terms of overall environmental impacts, compared to, for example, chemicals and reaction media related to cell growth and purification or biological waste which requires inactivation prior to disposal [23]. To that end, the application of a holistic, standardized methodology such as Life Cycle Assessment (LCA), taking into account the entire life cycle of biopharmaceuticals across multiple impact categories (e.g., Climate Change, ecotoxicity, resource depletion, and pollution of different environmental compartments), has the potential to provide important insights into the sustainability of these products, as well as to provide recommendations to manufacturers, in order to minimize their environmental impacts.

Studies have previously been performed to assess the environmental and cost impacts of insulin, including, for example, its production phase (e.g., [25–27]) and use phase (e.g., [28–30]). Nevertheless, a comparative assessment of different reagents and processes used in insulin production from a sustainability perspective is lacking. The objective of the present manuscript is to analyze the environmental impacts of some growth medium components commonly employed in the production of recombinant human insulin, using *E. coli* and yeast. Several scientific papers were consulted, describing different strategies to cultivate MOs for insulin production at the laboratory and pilot scales, and some representative papers were selected as the source of Life Cycle Inventory (LCI) data due to their detailed description of the materials, resources, and processes used. Considering that secondary sources of LCI data and assumptions were used in this study, it is not the objective to perform a complete comparison of different process alternatives from a sustainability perspective. Instead, different process inputs (i.e., reaction media) were compared, which may help guide bioprocess developers when selecting among material alternatives for insulin production. More importantly, a detailed description of the upstream processes, inputs, and outputs is given, along the value chain of each component that primarily contributes to the observed impacts. Such insights not only allow the selection of component alternatives that may lead to lower environmental impacts but can also help biopharmaceutical producers understand the link between elementary flows in LCA and environmental impacts of their final product. This can contribute to more informed decision making in the field of biopharmaceutical production. Furthermore, the results of the assessment reveal different hotspots of environmental impacts for different process alternatives, such as the carbon source, electricity use for autoclaving, and inducer for recombinant insulin production. Such insights allow the potential optimization, from a sustainability perspective, of different process alternatives, which target specific process steps and inputs and thus can significantly improve the environmental footprint of insulin production.

2. Materials and Methods

The literature research was performed using the Google Scholar platform between the 10 and 14 March 2025, using the following search terms: "insulin", "production", "synthesis", "recombinant", "*E. coli*", "*S. cerevisiae*", "*P. pastoris*", "upstream", "biopharmaceutical", and "fermentation". The retrieved scientific papers were evaluated in terms of level of detail reported regarding the experimental procedure, in order to select manuscripts that allow a complete LCI to be built. Furthermore, manuscripts were selected in order to cover a range of different upstream manufacturing process alternatives, i.e., different host MOs as biocatalysts, different strategies for the creation of the construct (proinsulin or separate production of the two chains), different reactor operation regimes (e.g., batch, fed-batch, continuous, etc.), and different scales of production. An overview of the selected manuscripts used to build the LCI for the present study is shown in Table 1.

Table 1. The selected scientific literature for Life Cycle Inventory (LCI) data collection.

Host MO	Reactor Operation	Reactor Size	Construct	Study Objective	Reference
E. coli	Cyclic fed-batch	5 L	Single chain	To increase the yield of insulin production by using a small fusion partner and C-chain peptide	[31]
E. coli	Batch	5 L	Single chain	To compare the production and purification of different human insulin precursors with various fusion peptides	[32]
E. coli	Fed-batch	50 L	Separate chains A and B	To avoid the use of chemical inducers by using a thermally inducible expression vector	[3]
P. pastoris	Batch	100 mL	Single chain	To optimize the induction by testing different starting inoculum densities, inducer concentrations, time points of induction, induction pH, and temperatures	[33]
S. cerevisiae	Fed-batch, followed by continuous (chemostat)	1 L	Single chain	To study production of a human insulin analog precursor in *S. cerevisiae* under long-term reactor operation and subsequent metabolic burden on the host MO	[34]

LCA was performed according to the corresponding standards [35,36], using the software SimaPro version 9.6.0.1 and LCI dataset library Ecoinvent version 3.10. The impact assessment was performed with the Environmental Footprint 3.1 method, and the results are expressed over 16 impact categories [37]. Cradle-to-Gate system boundaries were employed, starting from raw material extraction up to the preparation of growth media (cultivation, pre-cultivation, feed, and induction media). The process modeled here excludes the actual fermentation phase (i.e., bioconversion inside the bioreactor) and ends before product synthesis or any purification. The employed system boundaries are schematically presented in Figure 1. The focus of this work is to evaluate the impact of different growth medium components and, more specifically, to identify specific elementary flows and their contribution to the overall impacts. Therefore, the functional unit (and corresponding reference flow) of 1 L of medium was selected, and all inputs and outputs in the LCI were calculated in relation to this reference flow. For all components, the concentration (in grams per Liter or mols per Liter) was calculated, and the environmental impacts are reported accordingly. By expressing all calculated environmental impacts relevant to the concentration of components, a comparison of different microbial growth medium compositions is possible over different scales of production (i.e., from laboratory scale with medium volumes of 100 mL to the pilot scale, with medium volumes of several Liters).

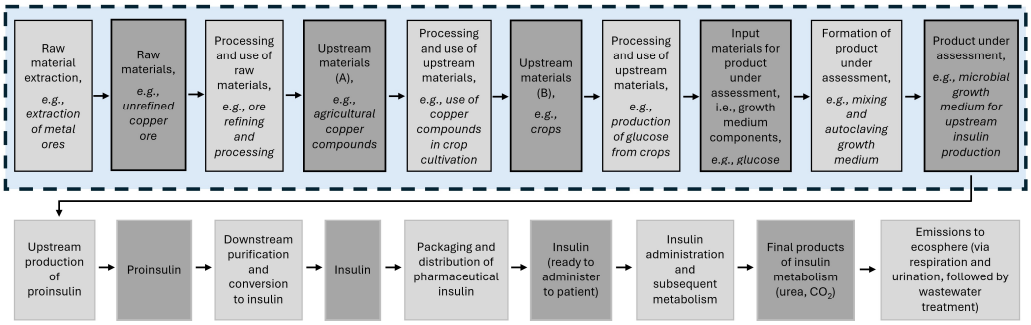

Figure 1. Schematic representation of the life cycle of recombinant insulin (cradle to grave), starting from the top left (raw material extraction) and following the arrows to the end (bottom right—emissions released to the environment at the End-of-Life stage). Light grey boxes indicate processes, whereas dark grey boxes indicate products (intermediate and final). The system boundaries are represented with dashed lines and include the processes and materials in the top row. As an example, the production and use of copper compounds, which are employed in crop cultivation in the upstream production of glucose, the latter being used as a carbon source for MOs during the production of insulin, is shown within the system boundaries.

For some of the materials and chemicals used in individual papers, information was not available to model their upstream manufacturing process, and therefore, proxies were used to model them in SimaPro. Specifically, for all the different vitamins used in various scientific papers, the production of vitamin D3 was used as a proxy, as reported by Morales-Gonzales and co-workers (2019) [38], using a batch large-scale process, with benzene as a solvent (i.e., scenario 3A in the corresponding report). Furthermore, for all antibiotics used in the included scientific papers used to construct the LCI here, the production of vancomycin hydrochloride was used as a proxy, as described by Ponder and Overcash (2010) [39]. The upstream production of the inducer IPTG (Isopropyl β-D-thiogalactopyranoside) was modeled based on reagents listed on the ChemicalBook website [40], while the amounts of reagents and products were modeled based on the synthesis of Isobutyl-C-galactoside (IBCG), a functional analog of IPTG [41]. The autoclaving of different reagents and growth media was modeled based on information from a recent impact evaluation report published by My Green Lab [42]. The electricity consumption of magnetic stirrers used during medium preparation was retrieved from the Joteo.net electricity usage calculator webpage [43]. It should be noted that for mixing, the device selected as a proxy was based on laboratory-scale equipment, and therefore, the relevance of the reported findings should be critically evaluated if large-scale insulin production is analyzed, wherein different equipment may be applicable. Finally, the composition of the trace element stock solution for *E. coli* cultures, unless described in the corresponding publication, was based on previously reported compositions used in bacterial bioreactors with mixed-culture facultative anaerobic microorganisms [44].

3. Results and Discussion

3.1. Understanding the Environmental Impact of Different Pre-Cultivation Growth Medium Preparations Used for E. coli

Different medium compositions have been examined for the pre-cultivation of *E. coli* strains with different plasmids carrying hybrid genes. In order to understand the impact of different medium components, the findings of Cho and co-workers (2001) [32], who examined four alternative options for plasmids in *E. coli*, grown using four different compositions of pre-cultivation media, were used. The compositions are shown in Table 2. Considering that the glucose concentration was not disclosed in some of the tested media,

only the medium used to cultivate *E. coli* JM109 with the pGEX-BA plasmid is analyzed, and the results are shown in Figure 2.

Table 2. Pre-cultivation growth medium compositions reported by Cho and co-workers (2001) [32].

pKBA Plasmid	pMAL-BA Plasmid	pGEX-BA Plasmid	pET-BA Plasmid
Yeast extract 0.5%	Yeast extract 0.5%	Yeast extract 1.0%	Yeast extract 1.0%
Tryptone 1.0%	Tryptone 1.0%	Tryptone 1.6%	Tryptone 1.6%
NaCl 1.0%	NaCl 1.0%	NaCl 0.5%	NaCl 0.5%
Glucose not disclosed	Glucose not disclosed	Glucose 2.0%	Glucose not disclosed
Ampicillin 50 μg/mL	Ampicillin 100 μg/mL	Ampicillin 100 μg/mL	Kanamycin 15 μg/mL

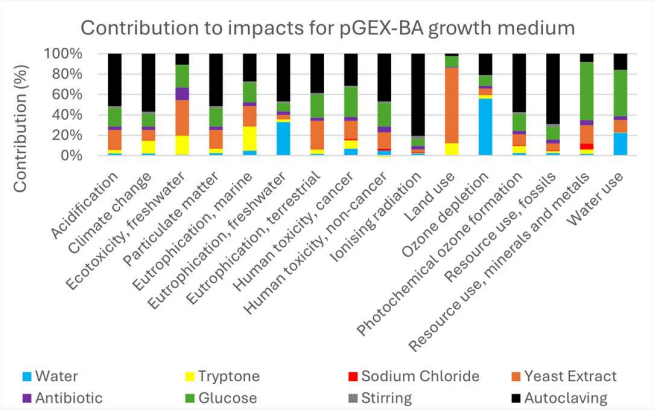

Figure 2. Contribution to environmental impacts of different growth medium components and preparation steps (i.e., autoclaving, stirring) for the medium used to cultivate *E. coli* JM109 with the pGEX-BA plasmid.

When examining the contribution of individual components and process steps per Liter of the pGEX-BA growth medium (Figure 2), it appears that the addition of glucose is, to an extent, responsible for its overall impacts per Liter of medium. This is primarily evident for the impact categories of Resource Use (Minerals and Metals) and Water Use, which exhibit the highest contribution of glucose to the total impacts (57% and 45%, respectively). At the same time, these impact categories also exhibit the largest increase in environmental impacts per Liter of medium, compared to the medium with the second highest overall impacts (i.e., medium used to cultivate *E. coli* BL21, harboring the pET-BA plasmid, analyzed excluding the glucose content, as it was not reported), corresponding to a 155% and 93% increase, respectively (results not shown).

For Resource Use of Minerals and Metals, the upstream process responsible for the majority of the observed impacts per Liter of *E. coli* JM109 growth medium is Tellurium extraction (45% of total Resource Use impacts per Liter of medium, with approximately 14% of the total impacts being associated with Tellurium depletion in the upstream production process of glucose). Among the processes contributing to Tellurium depletion in the value chain of growth medium production, 99% of the impacts are due to copper production (i.e., copper mine operation and beneficiation), likely associated with the use of copper in agriculture [45–47]. The highest contribution is from copper linked to the upstream production of glucose (54% of Tellurium depletion impacts) and yeast extract (19% of Tellurium depletion impacts), the latter modeled as fodder yeast (a co-product of ethanol production from whey fermentation) due to the absence of the exact product in the Ecoinvent library.

The agricultural upstream process for the cultivation of crops used to produce glucose and yeast extract further explains the high contribution of both components to the Land

Use impact category. Specifically, for yeast extract, which contributes to 73% of the total Land Use impacts per Liter of medium, the highest contribution to impacts (91% of all Land Use impacts per kg of yeast extract) is due to the production of cow milk, associated with the upstream production of fodder yeast (originating from whey, a by-product of cheese production). For glucose, with 11% contribution to the total Land Use impacts of the pGEX-BA growth medium, the majority of impacts are associated with the upstream production of maize grain (i.e., 10% of the total Land Use impacts per 1 L of the pGEX-BA growth medium).

Regarding the Water Use impact category, the majority of impacts (68%) per 1 L of pGEX-BA medium are due to irrigation. The highest contribution to the overall impacts is observed for the upstream production of glucose (45% of total Water Use impacts per Liter of medium) and upstream production of the yeast extract (12% of total Water Use impacts). Another process that significantly contributes to the Water Use impacts per Liter of pGEX-BA medium is autoclaving (16% of the total Water Use impacts per Liter of medium), primarily due to decarbonized water used for electricity production [48]. Finally, the ultrapure water used to produce the growth medium itself also has a significant contribution to the Water Use impact category, which corresponds to 22% for the pGEX-BA growth medium. Notably, ultrapure water also makes a measurable contribution to two other impact categories: Freshwater Eutrophication (33% of total impact per Liter of medium), and Ozone Depletion potential (56% of total impact per Liter of medium). Per 1 L of ultrapure water, Freshwater Eutrophication is primarily caused by phosphate emissions to water (over 99% of impacts), the majority of which are calculated based on the phosphate ion concentration difference between tap water and ultrapure water and modeled as emissions to water [49]. For Ozone Depletion, per one Liter of ultrapure water, the main contributor (over 93%) to impacts is CFC-113 (1,1,2-Trichloro-1,2,2-trifluoroethane) emissions into the air. These are linked to the process of reverse osmosis in the production of ultrapure water (93% of total impact per Liter of ultrapure water), associated with the use (and subsequent partial loss due to volatilization) of CFC-113 for the production of membranes utilized in reverse osmosis [50,51].

For the microbial growth medium composition shown in Figure 2, the autoclaving process significantly contributes to environmental impacts, particularly for the impact categories of Acidification, Climate Change, Particulate Matter, Eutrophication, Human Toxicity, Ionizing Radiation, Photochemical Ozone Formation, and Fossil Resource Use. Autoclaving was modeled taking into account the electricity used and the demineralized water used to operate the autoclave, per Liter of medium being autoclaved, in a device with 250 L of capacity. Different sub-processes and flows between the technosphere and the ecosphere were found to be responsible for the high impact of the autoclave process, particularly related to electricity production, which contributes to over 80% of the total impacts per 1 L of medium being autoclaved in every impact category (results not shown). For all aforementioned impact categories, electricity use contributes to over 99% of the impacts per Liter of medium being autoclaved. Specifically, for Acidification potential, the impacts of autoclaving are primarily due to nitrogen oxides (34% of total autoclave process impacts) and sulfur dioxide emissions into the air (65% of total autoclave impacts), primarily associated with the use of coal for electricity production [52,53]. Nitrogen oxide emissions into the air from electricity production are furthermore the main contributors to Photochemical Ozone Formation impacts, with a contribution equal to 77% of the total impacts per 1 L of medium being autoclaved.

For Climate Change, the main substance contributing to impacts is CO_2 emissions of fossil origin (89% of total autoclave impacts), primarily related to the use of coal for electricity production (over 60% of the total impacts from CO_2 emissions per Liter of

medium being autoclaved). For Particulate Matter (PM), the primary substance responsible is emissions of particulates < 2.5 μm (PM2.5 emissions) from electricity production (82% of total autoclave impacts). These are primarily associated with the coal mining upstream process for electricity production (corresponding to 80% of all impacts for PM2.5 emissions and 66% of total autoclave process impacts), as a result of different activities during coal mining, as has been previously reported [54]. In terms of Fossil Resource Use, different fuels used for electricity production are responsible for the observed impacts per Liter of medium being autoclaved, including, as main contributors, hard coal (44%), natural gas (26%), and uranium (17%). The use of nuclear energy in the global mix of electricity production, used here to model the high-voltage electricity in the autoclaving process, is also primarily responsible for the Ionizing Radiation impacts of the autoclave process. Specifically, per one Liter of medium being autoclaved, 99% of the impacts are due to Radon-222 and Carbon-14 emissions into the air, both of which are related to nuclear electricity generation. Over 97% of all Radon-222 emission impacts per Liter of autoclaved medium are the result of the treatment of Uranium milling tailings, and over 56% of all Carbon-14 emission impacts are the result of radioactive waste and spent nuclear fuel treatment, associated with the electricity use of autoclave operation.

The autoclaving process significantly contributes to Eutrophication potential over different environmental compartments, reaching 27% (for Marine Eutrophication), 47% (for Freshwater Eutrophication), and 39% (for Terrestrial Eutrophication). Different substances were shown to be responsible for these impacts when analyzing the contribution of water and electricity used to model the autoclave process per Liter of medium being autoclaved. For example, Marine Eutrophication and Terrestrial Eutrophication per Liter of medium being autoclaved are primarily (90% and 98%, respectively) due to nitrogen oxides emitted into the air during the upstream production of electricity. Freshwater Eutrophication impacts per Liter of medium being autoclaved are over 99% due to phosphate emissions to water during the upstream production of electricity and, more specifically, due to the treatment of spoil that is generated during the mining of lignite and hard coal (97% of total autoclaving process impacts), as has been previously reported [55–57]. Finally, the autoclaving process significantly contributes to the Cancer (32%) and Non-Cancer (49%) Human Toxicity potential. For Cancer Human Toxicity potential, the primary substance responsible (per Liter of medium autoclaved) was anthracene emissions into the air due to upstream electricity production (91% of total autoclave impacts). These are primarily due to upstream coke production (i.e., an industrial fuel produced from hard coal), employed in the generation of electricity (82% of total autoclave impacts due to coke production). The carcinogenic effects of coke production in general, and anthracene emissions in particular, have been previously described [58,59]. For Non-Cancer Human Toxicity of the autoclaving process, the primary substance responsible was Mercury (II) emissions into the air from the upstream production of electricity (63% of total autoclave process impacts). The impacts were primarily due to hard coal use for electricity generation (i.e., with hard coal contributing to over 70% of the Mercury (II) emissions' impacts and to over 40% of the total Non-Cancer Human Toxicity impacts of autoclaving, per Liter of medium). The contribution of hard coal use to Mercury emissions has been previously documented [60–62].

Different antibiotics were added to the growth media tested by Cho and co-workers (2001) [32]. Nevertheless, due to lack of LCA data for the production of different antibiotics, vancomycin HCl was used as a proxy for all different antibiotics reported in this study. Compared to other components of the growth media, antibiotics make a small contribution to the overall impacts of up to 12% (for pGEX-BA growth medium) in the impact category of Freshwater Ecotoxicity. The primary contributor (83% of total Freshwater Ecotoxicity

impacts per gram of antibiotic) is soybean meal, included as part of the LCI for vancomycin production [39]. The main substance responsible for the Freshwater Ecotoxicity impact is Chlorpyrifos, an organophosphate pesticide used in agriculture. The emissions of this substance to soil, water, and air result in 76% of the total Freshwater Ecotoxicity impacts per 1 g of produced antibiotic.

While the contribution of vancomycin to the total impacts of the growth media reported by Cho and co-workers [32] was relatively mild, other medium compositions described in the literature result in much higher contributions of the antibiotic due to the higher concentrations used. For example, the growth medium used by Shin and co-workers (1997) [31] contained 200 mg/L of antibiotic, which results in a significant contribution to the total environmental impacts per Liter of growth medium, ranging between 4% (for the Land Use impact category) and 37% (for the Freshwater Ecotoxicity impact category), as can be seen in Figure 3. Besides Freshwater Ecotoxicity, primarily due to Chlorpyrifos emissions, the antibiotic also significantly contributes to Mineral and Metal Resource Use (23%), Non-Cancer Human Toxicity (15%), Water Use (14%), and Cancer Human Toxicity (12% of total impacts per Liter of medium), with different substances and processes contributing to these impacts.

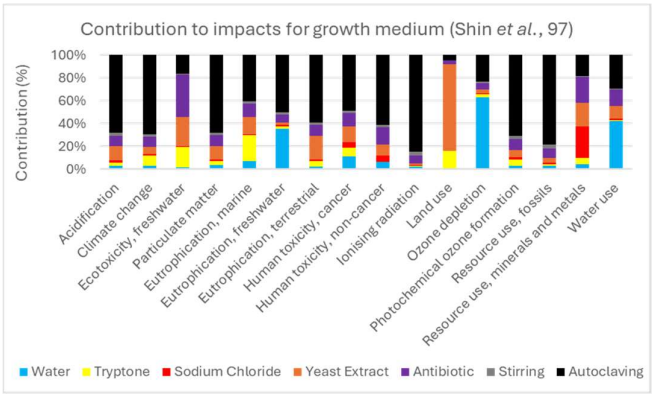

Figure 3. Contribution to impacts per Liter of pre-cultivation medium used for *E. coli* by Shin and co-workers (1997) [31].

Per gram of antibiotic, glucose used in the upstream production of vancomycin contributes to 69% of Mineral and Metal Resource Use, primarily due to copper mining and beneficiation for use in agriculture (42% of impacts per gram of antibiotic). Water Use impacts are also primarily caused by the upstream production of glucose (66% of total antibiotic impact), primarily due to irrigation for agricultural production of crops along the value chain of glucose (83% of total antibiotic impacts). Cancer Human Toxicity is primarily caused by glucose (45% of total antibiotic impacts), mainly due to coke production (40% of total antibiotic impacts), likely associated with heat and electricity production for use in glucose formation. Finally, Non-Cancer Human Toxicity is primarily the result of glucose (24%), electricity use (30%), and soybean meal (37% of total antibiotic impact). Overall, considering the significant contribution of the antibiotic to the impacts of the growth medium, and the contribution of different components along its upstream LCI to the overall impacts, it would be advisable to research the impacts of specific antibiotics used for insulin production on a case-by-case basis, in situations where an initial analysis with a proxy (as performed here) shows such significant contributions.

3.2. Understanding the Environmental Impact of Different Pre-Cultivation Growth Medium Preparations Used for Yeast

Pre-cultivation growth media were also described in studies using yeast as the host MO [33,34]. Here, MOs were first cultivated on agar plates, for which an assessment cannot

be performed due to lack of detailed inventory data for the amount and composition. From these cultures, small pre-cultures in flasks with liquid growth medium were inoculated and operated until they reached a certain optical density, and thereafter, the bioreactor was inoculated, and insulin production was induced. The composition of the pre-culture media was analyzed, and the results are shown in Figure 4. A comparative assessment of the media used for pre-cultivation of *P. pastoris* and *S. cerevisiae* is shown in Figure 4a. For this type of comparative assessment, the product (in this case, the growth medium) with the highest impact in each impact category is set to 100%, whereas other products with lower impacts are expressed as a percentage, compared to the highest one. Moreover, the individual contribution of different inputs per Liter for the two examined growth media is shown in Figure 4b,c.

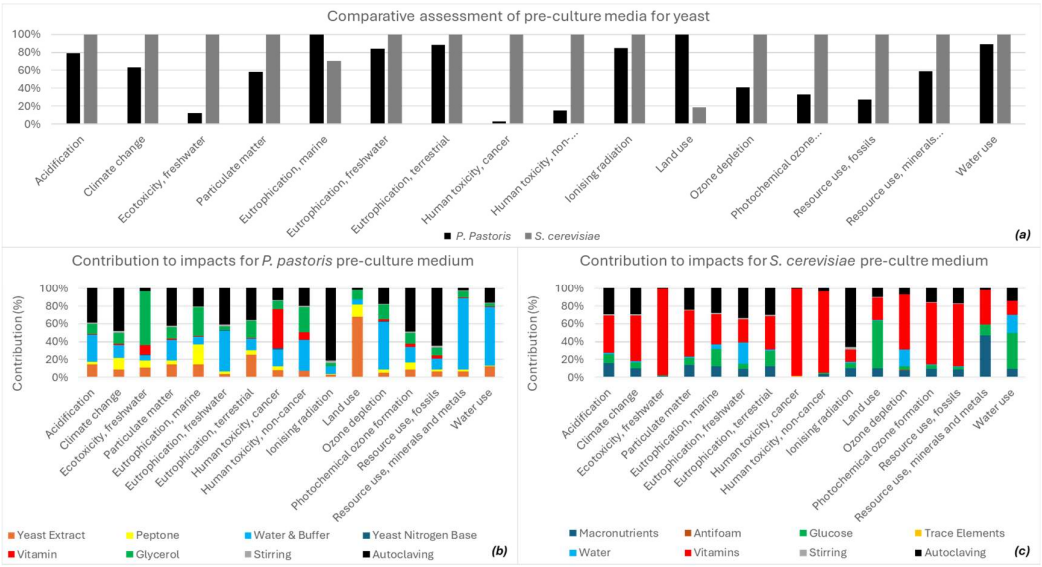

Figure 4. Environmental impacts of different yeast pre-culture growth media. (**a**) Comparative impacts of *P. pastoris* and *S. cerevisiae* pre-culture growth media. (**b**,**c**) Contribution to impacts for different components and process inputs for the production of 1 L of pre-culture growth medium used to cultivate *P. pastoris* (i.e., BMGY medium) (**b**) and *S. cerevisiae* (**c**).

As can be observed from the comparative assessment, the pre-culture medium used for *S. cerevisiae* has higher impacts in most impact categories, with the exception of Marine Eutrophication and Land Use (Figure 4a). For Marine Eutrophication, the main contributors per Liter of *P. pastoris* growth medium are glycerol (32%), peptone (22%), autoclaving (20%), and yeast extract (14%) (Figure 4b). In terms of the processes responsible for the impacts, the main contributor is the production of soybean (19% of total impacts per Liter of medium). Soybean is an input in the upstream synthesis of glycerol (modeled as glycerin and including esterification of various vegetable oils in its upstream production) and peptone (modeled as soybean meal). Further major contributors are rape seed production and alfalfa grass silage production (8% and 5%, respectively, per Liter of medium), both associated with the upstream production of yeast extract (modeled as fodder yeast produced from whey, a by-product of cheese production). Land Use impacts per Liter of *P. pastoris* medium are primarily due to cow milk and soybean production (over 61% and 14% of impacts per Liter of medium, respectively), associated with the production of yeast extract, peptone, and glycerol (67%, 14%, and 10% of impacts per Liter of medium, respectively, as can be seen in Figure 4b).

In addition to the contribution of glycerol to Marine Eutrophication and Land Use, discussed in the previous paragraph, a significant contribution per Liter of growth medium is also observed for the Ozone Depletion (17%), Terrestrial Eutrophication (19%), Non-Cancer

Human Toxicity (28%), and Freshwater Ecotoxicity potential impacts (58% of total impacts per Liter of pre-culture growth medium for *P. pastoris*, Figure 4b). Several substances were identified that contribute to the impacts of glycerol. For example, tetrachloromethane (CFC-10) emissions into the air, related to the upstream production of glycerol, were responsible for 12% of the total Ozone Depletion potential per Liter of medium. The processes responsible were shown to be chloroform production and gaseous chlorine production, both stemming from the upstream production of glycerol (27% and 13% of total CFC-10 emission impacts are due to these upstream processes within the glycerol value chain, respectively). This is likely due to the use of these compounds in the manufacture of pesticides and herbicides [63–66], in the upstream production of glycerol. Terrestrial Eutrophication per Liter of medium is partially due to ammonia emissions into the air, as part of the upstream process for glycerol production (13% of total medium impacts). These are primarily related to the upstream production of rape seed and soybean (30% of total ammonia emission impacts), as the contribution of agriculture to global ammonia emissions is well documented [67]. Non-Cancer Human Toxicity, as a result of upstream glycerol production, is partially due to emissions of Chlorpyrifos (10% of total impacts per Liter of medium) and Mercury (II) emissions to soil (7% of total impacts). The latter originate from Mercury contained in agricultural soils, either from natural or anthropogenic sources [68–70]. Finally, Chlorpyrifos emissions into the air, soil, and water from the upstream production of glycerol are also major contributors to Freshwater Ecotoxicity potential impacts per Liter of growth medium (i.e., 49% of total medium impacts due to these emissions).

Different hotspots can be identified in the overall impacts per Liter of growth medium used for *P. pastoris* (Figure 4b) and *S. cerevisiae* (Figure 4c). For example, ultrapure water, containing 100 mM potassium phosphate buffer (pH 6), contributes significantly to overall impacts of the *P. pastoris* pre-culture medium (ranging between 5% and 79%), particularly in the impact categories of Particulate Matter (22%), Acidification (29%), Non-Cancer Human Toxicity (34%), Freshwater Eutrophication (44%), Ozone Depletion (52%), Water Use (63%), and Mineral and Metal Resource Use (79%). Per Liter of buffer solution in ultrapure water, the majority of impacts in most impact categories are due to the potassium phosphate component (results not shown), ranging between 11% (for Ozone Depletion potential) and 99% (for Mineral and Metal Resource Use), whereas ultrapure water contributes significantly to Water Use (36%), Marine Eutrophication (43%), Freshwater Eutrophication (64%), and Ozone Depletion potential (89%). Considering the impact categories wherein the phosphate buffer solution contributes significantly to the growth medium impacts, different inputs and outputs are responsible for the observed impacts. For example, Water Use impacts are partially due to tap water consumption for the production of ultrapure water. A significant contribution is also observed for the potassium phosphate buffer component (modeled as sodium phosphate) and particularly the upstream production of phosphoric acid (over 45% contribution to the impacts per Liter of phosphate buffer solution), which has been previously identified as a water-intensive industrial process [71]. Ozone Depletion potential is primarily due to ultrapure water upstream production, particularly related to CFC-113 emissions. Finally, Freshwater Eutrophication potential is partially due to phosphate emissions to water as part of ultrapure water upstream production (64% of impacts per Liter of buffer solution) and partially due to the upstream production of the potassium phosphate buffer component (35% of impacts per Liter of buffer solution). The latter is primarily related to the landfilling of H_3PO_4 purification residue (17% of total phosphate emissions per Liter of buffer solutions), as part of the production process of phosphoric acid.

Several of the impact categories wherein the buffer solution makes a significant contribution to the overall impacts of the pre-culture growth medium are primarily affected

by the potassium phosphate components. For example, 94% of the Non-Cancer Human Toxicity impacts per Liter of phosphate buffer are due to the potassium phosphate compound and, more specifically, to lead (II), cadmium (II), and arsenic ion emissions into the air, contributing to 37%, 18%, and 17% of the total Non-Cancer Human Toxicity impacts per Liter of buffer solution, respectively. Lead emissions are primarily (over 60% of impacts for this compound) due to the upstream production of sulfuric acid used in the production of phosphoric acid [72]. This is due to the fact that sulfuric acid is modeled as a side product of copper concentrate smelting, a process known to result in significant emissions of lead and other heavy metals [73]. Similarly, the majority of impacts related to cadmium and arsenic emissions are also due to copper concentrate smelting, with up to 72% and 26% of impacts for each of these metal emissions being due to the upstream production of sulfuric acid and copper, respectively. Particulate Matter impacts per Liter of buffer solution are also primarily (91%) due to the potassium phosphate components, related to sulfur dioxide and PM2.5 emissions into the air (40% and 36% of total impacts per Liter of buffer solution, respectively). Furthermore, sulfur dioxide emissions into the air are also the primary cause of Acidification potential impacts per Liter of buffer solution (78% of total Acidification impacts per Liter of buffer), primarily linked to the upstream production of the potassium phosphate components (95% of Acidification impacts per Liter of buffer). Among the processes contributing to sulfur dioxide Particulate Matter emission impacts, the upstream production of sulfuric acid and sulfur is primarily responsible (over 69% of sulfur dioxide emission impacts). Approximately 49% of the PM2.5 emission impacts are due to the upstream production of electricity, heat, and diesel used along the value chain of the potassium phosphate component. Finally, the potassium phosphate component of the buffer solution is also primarily responsible for the Mineral and Metal Resource Use impacts per Liter of buffer (99% of total impact). This is primarily due to the depletion of Tellurium (47% of total impacts) during the upstream copper sulfide ore beneficiation (99% of total Tellurium depletion impacts), for the production of sulfuric acid used along the value chain of the potassium phosphate component.

Among the components of the *S. cerevisiae* pre-culture growth medium (Figure 4c), several hotspots can be observed for different impact categories. These include glucose (particularly in the categories of Water Use and Land Use, with a contribution of 40% and 53%, respectively), autoclaving (particularly for Ionizing Radiation, with a contribution of 66%), macronutrients (particularly for Mineral and Metal Resource Use, with a contribution of 47%), and vitamins, with a significant contribution in most impact categories, ranging from 14% (for Ionizing Radiation) to 98% (for Cancer Human Toxicity potential). Regarding vitamins, the growth medium described for *S. cerevisiae* pre-cultivation contained different vitamins (i.e., biotin, calcium pantothenate, nicotinic acid, inositol, thiamine HCl, pyridoxine HCl, para-aminobenzoic acid), at a total concentration of 29.25 mg/L. For all vitamins, the production of Cholecalciferol (vitamin D3) with benzene as a solvent, as described by Morales-Gonzalez and co-workers (2019) [38], was used as a proxy, while the authors also described additional production scenarios for vitamin D3, with different overall environmental impacts. For example, when the vitamin synthesis protocol based on isopropanol as a solvent (i.e., scenario 1B [38]) is used to model the impacts of the *S. cerevisiae* pre-culture medium, instead of benzene (i.e., scenario 3A [38]), the impact decreases for most impact categories, with an impact decrease ranging between 8% (for Freshwater Eutrophication) and 97% (for Cancer Human Toxicity). Instead, only a mild increase in impacts of up to 5% is calculated for Ionizing Radiation, Water Use, and Mineral and Metal Resource Use. Considering the significant contribution of vitamins to the overall impacts, and the large variation in impacts depending on the preparation protocol even for the same vitamin, it

is advisable to find more specific proxies for the vitamins used, to properly evaluate the impacts of biopharmaceutical production in future studies.

Regarding the macronutrients, the main contributor to Mineral and Metal Resource Use is ammonium sulfate, i.e., the second macronutrient in terms of concentration in the medium (i.e., 5 g/L, compared to 10.5 g/L for Potassium Hydrogen Phthalate). The primary substances responsible for Resource Use due to ammonium sulfate upstream production are lead (13%), silver (6%), and zinc (5% contribution to total Resource Use impact per Liter of growth medium). These impacts are primarily due to the origin of ammonium sulfate, as the market process used to model this compound contains different sources of ammonium sulfate, either via primary production from the reaction of ammonia and sulfuric acid or as a by-product of other processes. A percentage of the compound is formed as a co-product of the multi-output process of zinc production from concentrate, which includes several products such as zinc, lead, silver, copper, and gold [74]. Therefore, due to the allocation method applied to calculate the impacts per different product output, the produced ammonium sulfate carries part of the depletion impacts from metals contained in the zinc concentrate [75].

3.3. Comparative Assessment of Growth Media and Gene Expression Induction Strategies for E. coli and Yeast

The environmental impacts per Liter of medium used during cultivation of *E. coli* and yeast by different research groups were analyzed, and the results are shown in Figure 5a–f (for each medium) and Figure 5g (comparative assessment among all media). The media presented here were used to cultivate the host MOs for the production of recombinant insulin and contained different inducers employed in each study to trigger the expression of the target gene, such as IPTG (Isopropyl β-D-1-thiogalactopyranoside), employed by Shin and co-workers (1997) [31], and methanol, employed by Nurdiani and co-workers (2021) [33]. In addition to chemical inducers, other strategies were also investigated, such as the temperature-induced expression employed by Schmidt and co-workers (1999) [3]. For fed-batch experiments, different media are used over time during cultivation. These include the starting culture medium (i.e., the initial, nutrient-rich medium added to the bioreactor before inoculation with MOs, containing the essential components for early-stage growth and biomass accumulation) and the feed growth medium (i.e., medium supplied incrementally or continuously over time to the bioreactor, in order to replenish nutrients and sustain microbial growth without excessively diluting the culture volume). The composition and corresponding contribution to impacts of different medium components are shown for both the starting culture medium (Figure 5a,c) and the feed medium (Figure 5b,d).

In all medium compositions including trace elements (i.e., Figure 5a,c,d,f), it can be observed that their overall contribution to impacts is marginal and does not exceed 5% (observed for the Ionizing Radiation impact category of the culture medium employed by Shin and co-workers (1997 [31], Figure 5a). The trace elements typically employed in these compositions include the chelating agent EDTA (Ethylenediaminetetraacetic acid) and boric acid, as well as various metal salts, such as cobalt, manganese, copper, molybdenum, zinc, iron, iodine, and nickel. Trace elements are necessary for microbial growth in small concentrations [76,77], as opposed to macronutrients (including calcium, magnesium, phosphorus, sulfur, potassium, and sodium), which are needed in larger concentrations for microbial growth [78]. The impacts per mass of trace elements can be substantial, compared to more abundant metal salts included in the macronutrients. For example, the Mineral and Metal Resource Use impacts are 68 times higher for Sodium Molybdate compared to Magnesium Sulfate; the Ozone Depletion potential impact is 23 times higher for Potassium Iodide compared to Calcium Chloride; and the Non-Cancer Human Toxicity potential impact is 38 times higher for Copper Sulfate compared to Diammonium phosphate. However, the much lower concentrations employed in growth media for trace elements (i.e., in the range of mg/L, compared to g/L for macronutrients)

result in overall marginal impacts for the stock solution of trace elements per Liter of medium. Nevertheless, the contribution of trace elements should be evaluated on a case-by-case basis in future sustainability assessments of biopharmaceutical production, particularly considering that a proxy trace element composition was used in this analysis [44], in cases where the actual employed composition was not reported [31].

Figure 5. Environmental impacts of different cultivation/induction media investigated in this manuscript, for different MOs. (**a**,**b**) *E. coli* fed-batch cultivation medium (**a**) and feed medium (**b**) used by Shin and co-workers (1997) [31]. (**c**,**d**) *E. coli* fed-batch cultivation medium (**c**) and feed medium (**d**) used by Schmidt and co-workers (1999) [3]. (**e**) *P. pastoris* induction medium (i.e., BMMY medium with 2% methanol) employed in batch cultures by Nurdiani and co-workers (2021) [33]. (**f**) *S. cerevisiae* growth medium employed in fed-batch cultures by Seresht and co-workers (2013) [34]. (**g**) Comparative assessment of the aforementioned growth media.

Macronutrients, on the other hand, can make a significant contribution to the overall impacts per Liter of medium, as can be seen for the growth medium utilized by Schmidt and co-workers (1999 [3], Figure 5c). This is particularly evident for Non-Cancer Human Toxicity (22% of total impacts), Particulate Matter (26% of total impacts), Land Use (30% of total impacts), Water Use (31% of total impacts), Acidification (32% of total impacts), and Mineral and Metal Resource Use (68% of total impacts per Liter of medium). The major contributor to impacts in all the aforementioned impact categories is potassium phosphate (modeled as sodium phosphate here), with contributions to impacts per Liter of medium equal to 17%, 17%, 20%, 23%, 24%, and 50%, respectively. The substances and sub-processes along the value chain of potassium phosphate responsible for the majority of these impacts have already been discussed in Section 3.2, related to the impacts of the buffer solution used for the pre-cultivation of *P. pastoris*. Regarding Land Use, the process of phosphoric acid production is mainly responsible for the majority of impacts per kg of potassium phosphate (i.e., 72% of total impacts), likely due to phosphate mining [79] and the landfilling of generated waste from the upstream production of phosphoric acid [80].

In cases of fed-batch production, the starting culture medium typically has a more complex composition compared to the feed growth medium, as can be observed in the work of both Shin and co-workers (1997) [31] and Schmidt and co-workers (1999) [3]. Furthermore, an increased concentration of the carbon source (i.e., glucose) and yeast extract (i.e., a source of vitamins, proteins, and minerals for the growth of MOs) is typically utilized in the feed medium to sustain microbial growth in the fed-batch reactors. The higher concentrations of both glucose and yeast extract employed in the feed medium are responsible for the significantly higher impacts per Liter of medium of these components (Figure 5b,d), compared to the starting growth medium of the fed-batch reactors (Figure 5a,c). Similarly, the higher concentration of these components explains the overall higher impacts of the feed media compared to the starting media in most impact categories (Figure 5g). A similar trend was also observed between the batch growth medium and the chemostat growth medium for reactors operated by Seresht and co-workers (2013) [34], wherein the glucose concentration was increased from 20 g/L to 75 g/L, leading to an increase in environmental impacts in every impact category (results not shown), ranging from 1% increase (for Cancer Human Toxicity) to over 100% increase (for Land Use and Water Use impacts). On the other hand, higher impacts for some impact categories are observed for the *E. coli* starting growth medium used by Shin and co-workers (1997) [31], wherein the high concentration of vitamins (0.1 g/L of thiamine HCl) in the starting medium results in measurable impacts, compared to the feed medium.

Finally, the addition of the inducer to the feed or induction medium can result in a measurable increase in environmental impacts, depending on the compound used. For example, in the case of methanol, tested at a range of concentrations (from 1% to 5% v/v) as an inducer by Nurdiani and co-workers (2021) [33], the addition of the highest methanol concentration resulted in a measurable increase in impacts for most impact categories (results not shown), between 0.3% (for Land Use) and 52% (for Fossil Resource Use). A more significant contribution to impacts was observed for the IPTG inducer utilized by Shin and co-workers (1997) [31], wherein 1 mM IPTG addition to the growth medium resulted in up to 151 times higher impacts (for Ozone Depletion potential, results not shown), compared to growth medium without the component. It should be noted, however, that chemical inducers like IPTG are primarily used at the laboratory scale, while they are commonly replaced at commercial scales by alternative compounds such as lactose or methanol, as the latter have lower cost, can be metabolized and, therefore, do not accumulate after long-term operation, and do not result in toxicity at higher concentrations [81]. Therefore,

the relevance of these findings for upscaled production processes should be evaluated on a case-by-case basis, depending on the actual compounds used.

3.4. Towards Designing More Sustainable Growth Medium Formulations

In this work, several common components (e.g., nutrients, carbon source, etc.) and processes (i.e., autoclaving, stirring) employed in the preparation of microbial growth media for recombinant insulin production were analyzed in terms of their environmental impacts. Among the components examined, several hotspots of environmental impacts were identified, including the carbon source (i.e., glucose, glycerol) and commonly employed low-cost sources of nutrients (i.e., yeast extract). In agreement with the present findings, the environmental impacts of glucose production [82–84] as well as its high contribution to environmental impacts through microbial fermentation and biocatalytic processes [85,86] have been previously shown. An additional hotspot of environmental impacts over several impact categories was ultrapure water, i.e., water at purity levels required for pharmaceutical manufacturing [87,88], which has been previously identified as a hotspot of environmental impacts of bioprocesses and other industrial activities with requirements for high water purity [89–91]. Other commonly employed chemicals in the preparation of microbial growth media, such as potassium phosphate and ammonium sulfate, were also shown to significantly contribute to several impact categories, as has been previously shown for macronutrients in biocatalytic and microbial processes [92,93]. Both vitamins and antibiotics added to the growth media significantly contributed to several impact categories, in agreement with previous reports on growth medium components [94,95]. Finally, a significant contributor to several impact categories was the electricity consumption for the sterilization of the growth medium using an autoclave, in agreement with previous reports on the contribution of sterilization processes and electricity consumption to the overall environmental impacts of (bio)processes [95–97].

Such insights into the environmental impacts of different components of microbial growth media, as well as a more detailed understanding of the specific substances and subprocesses along the value chain of these inputs that result in significant impacts, may play an important role in optimizing microbial growth medium compositions from a sustainability perspective. For example, local, more sustainable sources of glucose and upstream production techniques could be selected by biopharmaceutical manufacturers [83,98,99] to decrease the overall environmental impacts of upstream production. Similarly, regarding yeast extract, which can be produced using a variety of substrates and processes [100–102], a more detailed evaluation of the sustainability aspects of different production techniques could help mitigate some of the impacts of insulin production, particularly taking into account the availability of local sources in the vicinity of biopharmaceutical manufacturing facilities. Water consumption in general, and the production and consumption of ultrapure water in particular, could become more sustainable by considering improved or alternative methods for the reclamation of wastewater from pharmaceutical production [103–106], potentially also combined with buffer recycling strategies from wastewater [107] or alternative methods for the production of ultrapure water [108–111]. While the reuse of water via wastewater treatment may not be a feasible solution for closed-loop recycling in the pharmaceutical sector at present, considering current Good Manufacturing Practices guidelines [106], such technologies may become viable in the future, particularly considering projected improvements in water purification technologies and the resulting quality of the reclaimed water [112–114]. Besides closed-loop recycling and the reuse of resources within the pharmaceutical industry, open-loop recycling and the use of reclaimed resources in other processes could also help reduce the environmental impact of the production of insulin and other biopharmaceuticals by avoiding the use of virgin materials and resources

outside the pharmaceutical industry. For other macronutrients, such as ammonium sulfate, upstream sources and their environmental impacts ought to be considered more carefully. A careful understanding of impact allocation processes in LCA and their practical applications and limitations is also necessary [115], particularly considering that the majority of the observed impacts were due to the co-production of ammonium sulfate as part of the multi-output process of zinc production. Regarding vitamins and antibiotics, more careful consideration of their environmental impacts on a case-by-case basis is crucial, considering their high contribution to the overall impacts of the examined growth media. Future work should focus on the development of detailed inventories for the specific compounds used to improve the reliability of the achieved LCA results. Finally, considering the electricity consumption for medium sterilization, biopharmaceutical manufacturers could consider strategies such as incorporating renewable electricity, recovering heat within their facilities [116], or utilizing more efficient sterilization techniques, thus improving energy efficiency [117,118]. Importantly, steam-based sterilization—commonly implemented at an industrial scale—could be considered as an alternative to electric-powered autoclaves [119,120]. Steam offers advantages such as high thermal efficiency and heat transfer, and its use at optimal capacity at a large scale (i.e., commercial production scale) may influence the overall environmental impact distribution, particularly in relation to the autoclaving stage [121].

4. Conclusions

In the present manuscript, several microbial growth medium compositions, typically employed in the upstream production of recombinant insulin, were analyzed in terms of environmental impacts, as well as the corresponding energy and water used for their preparation and sterilization (i.e., autoclaving, stirring). Different hotspots which contribute significantly to the overall environmental impacts per Liter of medium and the substances responsible were identified. Finally, comparative results were shown among different growth medium compositions in terms of environmental impacts. Overall, these findings can help researchers understand the processes and elementary flows along the value chain of growth medium components that are responsible for the observed environmental impacts. Based on such insights, researchers may select different sources of chemicals and feedstocks or employ strategies to decrease the concentration and to replace certain inputs with alternatives, which may bear lower environmental impacts.

By identifying high-impact components and evaluating alternative materials, this approach promotes informed decision making that aligns with both environmental and industrial goals. Integrating sustainability considerations at the formulation stage can significantly reduce the ecological footprint of biobased processes, fostering a more responsible and resilient biopharmaceutical industry. In particular, by considering microbial growth medium formulations at different production scales, including the Research and Development phase at the laboratory scale, this analysis highlights the value of integrating environmental impact assessments early in the development of microbial growth media for biopharmaceutical production. At this stage, there is still significant design flexibility, allowing for the selection and optimization of components with lower environmental burdens, before processes are locked in and scaled up. On the other hand, considering that the Life Cycle Inventory data employed in this study is based on secondary sources (i.e., scientific literature), primarily at the laboratory scale, the relevance of the reported findings for large-scale biopharmaceutical production ought to be evaluated on a case-by-case basis by industry experts, with detailed knowledge of confidential growth medium compositions at industrially relevant scales. Nevertheless, by embedding sustainability considerations from the outset, such assessments support the development of more environmentally re-

sponsible biobased processes and facilitate long-term improvements in resource efficiency and environmental performance.

Author Contributions: Conceptualization, K.-R.C., A.P., F.P., and E.P.K.; methodology, K.-R.C. and A.P.; software, K.-R.C. and A.P.; validation, K.-R.C., A.P., F.P., and E.P.K.; formal analysis, K.-R.C.; investigation, K.-R.C. and A.P.; resources, K.-R.C., A.P., and F.P.; data curation, G.A. and E.P.K.; writing—original draft preparation, K.-R.C. and A.P.; writing—review and editing, G.A., F.P., and E.P.K.; visualization, K.-R.C., F.P., and E.P.K.; supervision, E.P.K.; project administration, E.P.K.; funding acquisition, E.P.K. All authors have read and agreed to the published version of the manuscript.

Funding: The activities presented in this paper were carried out as part of the European Union's Horizon 2020 research and innovation programme ENVIROMED: "ENVIROMED—Next generation toolbox for greener pharmaceuticals design & manufacturing towards reduced environmental impact" (Grant Agreement no. Project 101057844).

Data Availability Statement: Dataset available on request from the authors.

Conflicts of Interest: Author Konstantina-Roxani Chatzipanagiotou, Athanasios Pappas, Foteini Petrakli, George Antonaropoulos and Elias P. Koumoulos were employed by the company IRES—Innovation in Research and Engineering Solutions SNC. The authors declare that the research was conducted under the framework of the EU-funded project "ENVIROMED", in the absence of any commercial or financial relationships that could be construed as potential conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

LCA	Life Cycle Assessment
E. coli	*Escherichia coli*
S. cerevisiae	*Saccharomyces cerevisiae*
P. pastoris	*Pichia pastoris*
MO	Microorganism
LCI	Life Cycle Inventory
IPTG	Isopropyl β-D-thiogalactopyranoside
IBCG	Isobutyl-C-galactoside
CFC-113	1,1,2-Trichloro-1,2,2-trifluoroethane
CFC-10	Tetrachloromethane
EDTA	Ethylenediaminetetraacetic acid
PM	Particulate Matter

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