Review

Advances in Concentration Gradient Generation Approaches in a Microfluidic Device for Toxicity Analysis

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Abstract: This systematic review aimed to analyze the development and functionality of microfluidic concentration gradient generators (CGGs) for toxicological evaluation of different biological organisms. We searched articles using the keywords: concentration gradient generator, toxicity, and microfluidic device. Only 33 of the 352 articles found were included and examined regarding the fabrication of the microdevices, the characteristics of the CGG, the biological model, and the desired results. The main fabrication methods were soft lithography, using polydimethylsiloxane (PDMS) material (91%) and SU-8 as the mold (58.3%). New technologies were applied to minimize shear and bubble problems, reduce costs, and accelerate prototyping. The Christmas tree CGG design and its variations were the most reported in the studies, as well as the convective method of generation (61%). Biological models included bacteria and nematodes for antibiotic screening, microalgae for pollutant toxicity, tumor and normal cells for, primarily, chemotherapy screening, and Zebrafish embryos for drug and metal developmental toxicity. The toxic effects of each concentration generated were evaluated mostly with imaging and microscopy techniques. This study showed an advantage of CGGs over other techniques and their applicability for several biological models. Even with soft lithography, PDMS, and Christmas tree being more popular in their respective categories, current studies aim to apply new technologies and intricate architectures to improve testing effectiveness and reduce common microfluidics problems, allowing for high applicability of toxicity tests in different medical and environmental models.

Keywords: microfluidic device; microdevice; concentration gradient generator; CGG; toxicity; drug screening; microdevice gradient generator

1. Introduction

The toxicological assessment of chemicals, pharmaceuticals, food and food ingredients, cosmetics, and industrial products has significantly advanced due to scientific and technological developments. New techniques, such as the promising alternative of human-cell seeded organ-on-chips for acute systemic toxicity, as well as in silico approaches, have been replacing conventional techniques, for example, tests which use LD50 as their main parameter, requiring a great number of animals to determine the chemical dose able to achieve 50 percent of deaths [1]. After the 1980s, researchers were encouraged to modify their experimental design strategies in order to reduce, refine, and also replace (3Rs) the
conventional methods including animal experimentation. The 3Rs principles led to a dramatic decrease in the use of animals in research and development while also lowering the failure rate of pharmaceuticals [2].

When compared to in vivo studies, in vitro studies demonstrated time and financial savings, high yield, high reproducibility, and fewer ethical concerns [3]. As a result of their advancement and technological innovations, the microfluidic device was able to be created, opening up new possibilities, allowing the association of multiple components, and functioning as a “mini laboratory”, also known as a “lab-on-a-chip”, with possible application in areas such as chemistry, environment, bioenergetics and health [4–6].

The fabrication of microfluidic devices requires a set of procedures that enable the development of structures at a micrometric scale with great precision, in order to ensure a laminar flow of fluid in the microchannels [7]. The soft lithography technique is widely used for stamping or micromolding processes due to its ease, effectiveness, and low cost [8]. Complementary techniques, however, have been investigated for the creation of microdevices, which ensures a wider variety of possibilities for the employment of various polymers and structures. These techniques include photolithography and stereolithography [9].

The concentration gradient generator (CGG) is a type of microfluidic device capable of generating a concentration gradient via convection-mixing-based (tree-shape and altered-tree-shape), laminar-flow-diffusion-based (Y-shape), membrane-based, pressure-balance-based, droplet-based, and flow-based methods. All of these different techniques have been proposed and evaluated in a variety of experiments, allowing the study of numerous biological processes, such as cell migration, immunological response, wound healing, cancer invasion and metastasis, inflammation and chemotaxis, and the investigation of the concentration at which an element becomes harmful to an organism [10]. Compared to traditional macroscale evaluation methods, CGG microdevices allow for high analysis performance, with low reagent consumption, more efficient use of samples with limited volumes, a high surface-to-volume ratio, spatio-temporal resolution, portability and easy customization, control, and automation [11,12].

The CGG microdevice technology combines the advantages of microfluidics with a three-dimensional (3D) cell compartment that can preserve the biological complexity of cell models (3D cultures, including microenvironment or vascularization) and mimic drug evaluation, similar to animal models. Many drug candidates in different concentration ranges are evaluated at the same time, and different treatment regimens can also be explored using multiple drug gradient generators and parallel cell culture chambers [13].

The demand for novel medication development is at an all-time high, due to rising drug resistance and the emergence of new diseases, motivating the search for more efficient drug screening methods. The CGG microdevice approach to performing the antimicrobial susceptibility test (AST) is a simple, economic, and fast way to emulate a traditional AST and rapidly provide the minimal inhibitory concentration (MIC) of an antibiotic for a certain bacterial strain, at rates comparable to those of other miniaturized devices and automated AST instruments. The MIC value allows clinicians to prescribe appropriate dosages of the medication and stop bacteria from becoming resistant before being eradicated [14].

In this systematic review, our objective was to investigate how studies have applied micro-CGG for toxicological evaluation and for what purposes, in addition to the technological evolution in the development of these systems. The microfluidic device manufacturing and new technologies applied, the perspective of design and methodology of the CGG system, and the type of biological environment used to evaluate the substance’s toxicity, as well as the outcomes, were considered.

2. Materials and Methods
2.1. Search Strategy

We conducted a systematic search for articles that were published in the previous 10 years, including the years between 2011 and 2022. The articles selected, which are indexed in PubMed and Scopus, followed the Preferred Reporting Items for Systematic
Reviews and Meta-Analyses (PRISMA) guidelines [15]. The criteria of interest selected were keywords in the following sequence: ((Concentration Gradient Generator) AND (Toxicity) AND (Microfluidic Device)), using the boolean operators (DecS/MeSH):

SCOPUS: (((TITLE (“organs-on-chips”) OR TITLE (“organs-on-a-chip”) OR TITLE (“microfluidic device”) OR TITLE (“lab-on-chips”) OR TITLE (microfluidics) OR TITLE-ABS-KEY (microfluidic)) AND PUBYEAR > 2010 AND PUBYEAR > 2010) AND ((SRCTITLE (toxicity) OR TITLE (toxicities) OR SRCTITLE (toxicological) OR TITLE (nanotoxicity)) AND PUBYEAR > 2010 AND PUBYEAR > 2010)) OR (((TITLE (“concentration gradient generator”) OR TITLE-ABS-KEY (“microfluidic gradient generator”)) AND PUBYEAR > 2010 AND PUBYEAR > 2010) AND ((TITLE (“organs-on-chips”) OR TITLE (“organs-on-a-chip”) OR TITLE (“microfluidic device”) OR TITLE (“lab-on-chips”) OR TITLE (microfluidics) OR TITLE-ABS-KEY (microfluidic)) AND PUBYEAR > 2010 AND PUBYEAR > 2010)) OR ((((((“organs-on-chips”(Title)) OR (“organs-on-a-chip”(Title))) OR (“microfluidic device”(Title))) OR (“lab-on-chips”(Title))) OR (microfluidics(Title))) OR (microfluidic(Title)) AND (2011/1/1:2022/6/1(pdat))) AND (((Concentration Gradient Generator”(Title/Abstract)) OR (“microfluidic gradient generator”(Title/Abstract)) AND (2011/1/1:2022/6/1(pdat)))) Filters: from 1 January 2011 to 6 June 2022).

2.2. Selection Criteria

We only included original articles written in English published within the previous 10 years that used a microfluidic device capable of generating a gradient to analyze the toxicity of different concentrations of a substance to living organisms. The selection factors were in accordance with the PICO criterion we used: Problem: difficulty in generating a linear concentration gradient of a substance quickly and effectively; Intervention: use of microfluidics device to generate gradients; Comparison: to assess substances’ toxicity screening with concentrations generated by CGG and by pipetting; Outcome: toxicity assessment.

2.3. Exclusion Criteria

The following exclusion criteria were used: (i) reviews, (ii) publications written in languages other than English, (iii) indexed articles published in more than one database (duplicates), (iv) only microdevice fabrication protocols, (v) does not assess the toxicity effect in biologic organism, (vi) does not apply toxicology test, and (vii) the microdevice did not employ a concentration gradient generator.

2.4. Data Compilation

In this review, eight of the authors (N.M.E.V., M.P.N., A.H.A., L.D.R., J.B.M., F.A.O., C.S.L., A.T.L., M.N.P.C., and L.F.G.), in pairs, independently and randomly analyzed, reviewed, and assessed the eligibility of titles and abstracts according to the strategy of established search. The authors N.M.E.V., M.P.N., A.H.A., L.D.R., J.B.M., and L.F.G. selected the final articles by evaluating the texts that met the selection criteria. The authors N.M.E.V., L.D.R., J.B.M., F.A.O., C.S.L., and L.F.G. were responsible for the search for the characteristics and fabrication of the CGG with the collaboration and review of the authors A.T.L. and M.N.P.C. The authors N.M.E.V., M.P.N., A.H.A., L.D.R., and L.F.G. searched for the device microenvironment and toxicity techniques. All authors contributed to writing the entire text of this review.

2.5. Data Extraction

Four topics were used to analyze the papers under review, and they were represented in tables that addressed the following features: (1) characteristics, design, and fabrication of
concentration gradient generator microfluidic devices for toxicity analyses; (2) microfluidic concentration gradient generators’ characteristics; (3) biological model used for toxicity evaluation; and (4) outcomes of the studies.

2.6. Risk of Bias Assessment

The selection of articles was performed in 2 pairs, and, in case of disagreement, an independent senior author decided on whether the article in question would be included. The data selected in the tables were divided by the authors into the groups already described above, and the checking of the data was carried out by the following group. In the case of disagreement, author L.F.G. made the final decision.

2.7. Data Analysis

The data obtained in each of the tables were analyzed in percentages and range distribution to highlight the main characteristics, particularities, and exceptions, according to applicability.

3. Results

3.1. Selection Process of the Articles Identified According to the PRISMA Guidelines

We searched the PubMed and Scopus databases for publications from the last 10 years, considering the period from 2011 to March 2022 and following the selection inclusion and exclusion criteria already presented, resulting in 352 articles identified, comprising 254 articles from Scopus and 98 from PubMed. Of the 254 articles found in Scopus, 86 were excluded after screening because 31 were reviews, 43 were conference papers, 8 were book chapters, and 4 were not eligible. At screening, 56 articles from PubMed, comprising 47 duplicates and 9 reviews, were also excluded. Eligibility analysis was carried out following the selection criteria, and 139 of the 168 articles from Scopus (45 did not report the organism used in the study, 26 only reported the device development, 30 did not assess toxicity, 17 did not apply the concentration gradient generation in the device, and in 21 the toxicity was assessed outside the device) and 38 from PubMed (8 did not report the organism used in the study, 16 only reported the device development, and 14 did not assess toxicity) were excluded. Thus, only 33 unduplicated full-text articles [16–48] were included in this systematic review, 29 from Scopus and 4 from PubMed, as shown in Figure 1.

The 33 selected studies were analyzed regarding the microdevice fabrication, the CGG characteristics, the biological model, and the main outcomes. Due to the different biological approaches of the selected studies, the tables were organized internally by the four main types of organisms used for toxicity analysis inside of the device: 7 studies used microorganisms (bacteria and nematodes) (21%) [16–22], 5 used microalgae (15%) [23–27], 19 used tumor cells and other models (58%) [28–46], and 2 used zebrafish embryos (6%) [47,48].

3.2. Characteristics, Design, and Fabrication of Concentration Gradient Generator Microfluidic Devices for Toxicity Analyses

The technologies utilized in the design, manufacturing, finishing, and innovations of microfluidic devices to generate concentration gradients for toxicological analysis and drug screening in the studies included in this systematic review are summarized in Table 1. General analysis was initially performed regarding the date of publication of the 33 articles with the division performed according to the organisms used for the toxicity assessments (microorganisms, microalgae, tumor cells and other models, or zebrafish embryos), pointing out that in the studies carried out in the last 5 years [16–19,23,24,28–34], the use of microorganisms and tumor cells and other models was more prominent, showing a higher incidence and demand for microdevices focused on efficient drug screening. This specific division by the biological model of Table 1 did not necessarily have a connection with particularities in the manufacturing techniques related to the microfluidic characteristics.
Figure 1. Schematic representation of the process for articles’ identification, screening, and eligibility for inclusion in this systematic review following the PRISMA guidelines.
Table 1. Characteristics, design, and fabrication of concentration gradient generator microfluidic devices for toxicity analyses.

| Study           | Year | Material | Layers | Technology       | Geometry                                                                 | Mold                  | Device Assembly                  | Manufacturing | New Technologies                                                                 |
|-----------------|------|----------|--------|------------------|---------------------------------------------------------------------------|-----------------------|-----------------------------------|---------------|---------------------------------------------------------------------------------|
| Zeng, W. et al. | 2022 | PDMS     | 2      | Soft lithography | Chambers: 500 × 300 × 30 µm (L × W × H); Spacing between chamber: 40, 70, 100, 130, 160, and 190 µm | SU-8                  | UV photolithography              | Glass         | O₂ plasma                          | In-house                                               |
| Nagy, K. et al. | 2022 | PDMS     | 3      | Soft lithography | Upper layer: two trapezoid-shaped reservoirs (sides: 1.0, 0.5, 0.5, 0.65 cm (L), and 45 µL (Vol)); bottom layer: 0.04 × 1.2 × 10 mm (H × W × L), and 0.4 µL (Vol); overlapping area: 100 µm² | SU-8                  | UV photolithography              | Glass         | Partially cured PDMS               | In-house                                               |
| Sweet, E. et al.| 2020 | Polymer  | NA     | 3D printing      | Integrated vertical µ-mixers and channels between layers: 5000 × 7500 µm (L × D); bulbs: 1250 µm (D) | NA                    | NA                                | NA            | NA                                | In-house                                               |
| Tang, M. et al. | 2018 | PDMS     | 3      | Soft lithography | Capillary valve: 2 × 0.2 × 0.3 mm (L × W × H); metering chambers: 10 µL (Vol), 2.5 × 4 × 1 mm (L × W × H); inlet and outlet: 2 × 2 mm (D × H); ITO electrodes: 0.8 mm (W), 1350 A (H), 0.8 mm (spacing) Narrow path: 10 µm; PDMS on glass cover: 100 µm (T) | PMMA                  | CNC machining                    | PDMS          | Partially cured PDMS               | In-house                                               |
| Zhang, B. et al.| 2014 | PDMS     | 3      | Soft lithography | Mixers: 200 × 60 µm (W × H); eight T-shaped channels: 280 × 60 µm (W × H); eight ellipses observing chambers: 3 × 4 × 0.5 mm (minor axis L × major axis L × H); inlet and outlet: 2 × 2 mm (D × H); ITO electrodes: 0.8 mm (W), 1350 A (H), 0.8 mm (spacing) Narrow path: 10 µm; PDMS on glass cover: 100 µm (T) | SU-8                  | UV photolithography              | PDMS / ITO glass   | O₂ plasma                          | In-house                                               |

Uses the properties of diffusion of gases based on vacuum pressure levels for drug gradient formation

Use of a porous membrane for the diffusion of molecules from one reservoir to the other

Unconventional 3D printing manufacturing with multidrug testing capability

Unconventional use of centrifugal microfluidics for the CGG; includes a laser photodetector and optical power meter

Integration of an ITO glass layer for impedance system for worm-counting control
### Table 1. Cont.

| Study                  | Year | Material | Layers | Technology          | Microfluidic Device | Mold | Device Assembly | Manufacturing | New Technologies |
|------------------------|------|----------|--------|---------------------|---------------------|------|-----------------|---------------|------------------|
| DiCicco, M. and Neethirajan S. [21] | 2014 | PDMS     | NR     | Soft lithography    | Gradient mixing module: $20 \times 100 \times 18,750 \, \mu m$ (H × W × L) and the observation module: $20 \times 1000 \times 12,000 \, \mu m$ (H × W × L); bacteria inlet channel: 50 µm (W); inlet and outlet holes 750 µm (D) | SU-8   | UV photolithography | Glass | NR | In-house | Vertical microchannel used for bacterial inoculum diffusion evaluation |
| Yang, J. et al. [22]    | 2013 | PDMS     | 2      | Soft lithography    | Top layer: central reservoir (2.5 × 1 mm (D × H)) and eight inlets (2.0 × 1 mm (D × H)); Bottom layer: channels and culture chambers (2.0 × 0.5 mm (D × H)). Each channel is connected to a chamber by a thin “gate sill” (40 × 40 × 30 mm (W × H × T)) | Glass plate/copper plate | CNC machining | PDMS | O₂ plasma | In-house | Worm dispenser system CGG microintegration |

**Microalgae**

| Study                  | Year | Material | Layers | Technology          | Microfluidic Device | Mold | Device Assembly | Manufacturing | New Technologies |
|------------------------|------|----------|--------|---------------------|---------------------|------|-----------------|---------------|------------------|
| Wang, Y. et al. [23]   | 2019 | PDMS     | 1      | Soft lithography    | Specifications: 250 µm (sheath (center) inlet), 1125 µm (sample (side) inlet W), 1 mm (symmetrical micropost array W), 32 mm (overall L), 35 µm (vertical and horizontal spacing between the microposts), 50 µm (post D), 60 µm (channel H) | SU-8   | UV photolithography | Glass | NR | In-house | Use of the principle of DLD for the separation of microalgae in the system |
| Han, B. et al. [24]    | 2019 | PDMS     | 4      | Soft lithography    | Microchannels: $100 \times 100 \, \mu m$ (deep × W); culturing chambers: $2 \times 1.2 \, mm$ (L × W). The parallel channels and culturing chambers are 30 µm apart from each other and connected by diffusion channels (5 µm in depth) | SU-8   | UV photolithography | PDMS | Plasma | In-house | Combinational–mixing–serial dilution design used to generate parallel gradients for mixing chemicals (binary/ternary/quaternary mixture) using an algorithm |
### Table 1. Cont.

| Study                          | Year | Material | Layers | Technology       | Geometry                                                                 | Mold                | Device Assembly | Manufacturing | New Technologies |
|-------------------------------|------|----------|--------|------------------|---------------------------------------------------------------------------|---------------------|-----------------|---------------|------------------|
| Zheng, G. et al. [25]         | 2014 | PDMS     | 1      | Soft lithography | Reservoir: 5 mm (D); outlet holes: 1 mm (D); diffusible chamber connected between two parallel channels of each outlet of CGG: 500 µm (W), 2 mm (L); Channels and chambers: 50 µm (distance apart), 50 µm (H); chamber connected to flow channels by diffusion channels: 50 µm (W), 30 µm (distance apart), 2 µm (H) | SU-8 UV photolithography | PDMS Air plasma | In-house | NR |
| Zheng, G. et al. [26]         | 2013 | PDMS     | 3      | Soft lithography | Three layers: the bottom flow layer containing flow channels to form an upstream CGG (100 × 50 µm (W × H)) and downstream parallel diffusion channels (1.2 × 2 × 0.1 mm (W × L × H)); polycarbonate membrane (10 µm (T), 1.2 µm (D pores)); the top culturing layer has structures of 16 isolated dead-end chambers for microalga culture and imaging (l: (1.2 × 2 (W × L), hl: 100 µm (H)) | SU-8 UV photolithography | PMMA Partially cured PDMS/O<sub>2</sub> Plasma | In-house | NR |
| Zheng, G. et al. [27]         | 2012 | PDMS     | 2      | Soft lithography | Three cell culture chambers are connected between two parallel channels of each outlet of the CGG. The channels and chambers: 25 × 60 µm (distance apart × H), flow channels: 200 µm (W), chambers: 1.2 × 2 mm (W × L). The flow channels and chambers are connected by diffusion channels: 200 × 400 × 3 µm (W × L × H) | SU-8 UV photolithography | PDMS Air plasma | In-house | 3D culture system indirectly integrated by diffusion to the CGG |
| Study                        | Year | Microfluidic Device | Mold | Device Assembly | Manufacturing | New Technologies                                                                 |
|-----------------------------|------|---------------------|------|-----------------|--------------|-----------------------------------------------------------------------------------|
| **Tumor cells and other models** |      |                     |      |                 |              |                                                                                   |
| Chennampally, P. et al. [28] | 2021 | Silicon 1           | NA   | NA              | In-house     | Cell culture chamber: 1 × 1 × 0.1 mm (L × W × H); the cell culture chamber is covered with a thin glass cover slip ≈ 3 × 3 × 0.17 mm (L × W × H) Anodic bonding; biomedical-grade RTV adhesive Unconventional material that avoids chemical absorption and leaching problems introduced by PDMS |
| Yin, L. et al. [29]         | 2020 | PDMS OOC: 3 CGG: 2  | PMMA | Laser cutting   | In-house     | Kidney channel: 0.5 × 1 mm (W × H), cell culture: 14 mm (D); CGG- NR O2 plasma Direct interaction of an OOC with its own temperature control with a CGG |
| Jaberi, A. et al. [30]      | 2020 | PDMS 2              | Etched silicon | UV direct writing photolithography | In-house | Microchambers for both devices: 1 mm (D); micropillar array: 80 µm (D of each pillar) O2 plasma Use of hydrostatic pressure to conduct the fluid flow, with a pump-free microfluidic gradient generator |
| Zhao, X. et al. [31]        | 2019 | PDMS 2              | SU-8 | UV photolithography | In-house | Channel: 25 × 200 µm (H × W); culture chamber: 5850 µm (W) O2 plasma Innovation in using two different integrated chips for CSE concentration generation |
| Qin, Y.X. et al. [32]       | 2018 | PDMS 2              | NR   | NR              | In-house     | Chip A: main channels and cell chambers 2 × 0.1 mm (D × H); Chip B: cell chambers 2 × 1 × 0.1 mm (L × W × H), central channel: 15 × 0.8 × 0.1 mm (L × W × H), traffic channels: 30 × 100 µm (W × H) O2 plasma Innovation in using two different integrated chips for CSE concentration generation |
| Luo, Y. et al. [33]         | 2018 | PDMS 2              | SU-8 | UV photolithography | In-house     | NR                                                                 |

Table 1. Cont.
| Study                          | Year | Material      | Layers | Technology     | Geometry                                                                 | Mold Material | Fabrication    | Cover          | Sealing         | Manufacturing       | New Technologies                           |
|-------------------------------|------|---------------|--------|----------------|--------------------------------------------------------------------------|---------------|----------------|----------------|----------------|------------------|---------------------------------------------|
| Lim, W. and S. Park [34]      | 2018 | PDMS          | 2      | Soft lithography | Chip dimensions: 4 × 3 × 0.8 cm (L × W × H); top layer: 6 mm (T); two inlets: 8 mm (D); gradient generator: microchannels 150 µm, culture array with fifty cell injection holes 700 µm (D) and five outlets 2 mm (D); bottom layer: 2 mm (T) with 50 concave microwells 400 × 200 µm (D × H) | SU-8          | UV photolithography | PDMS          | O₂ plasma      | In-house         | Integration of µFSCD with a CGG             |
| Jin, D. et al. [35]           | 2016 | PDMS          | 3      | Soft lithography | Inlet and outlet: 1.2 mm (D)                                              | SU-8          | UV photolithography | Glass         | O₂ plasma      | In-house         | Layer combination for the integration of spheroid cell culture and 2D culture with interaction over a porous membrane |
| Hong, B. et al. [36]          | 2016 | PDMS and Paper plastic | 3 | Soft lithography | Inlets and outlets: 6 mm (D); 2 mm (channel W); chip dimensions: 49 × 47 mm (L × W) | SU-8          | UV photolithography | Top and bottom plastic | Screw fastening | In-house         | Paper-based chip                               |
| Ying, L. et al. [37]          | 2015 | PDMS          | 3      | Soft lithography | CGG: inlets 1.5 mm (D); microchannels 10 × 0.2 × 0.1 mm; cell culture chambers: 800 × 400 × 100 µm (L × W × H); inlet and outlet: 0.6 mm (D); upper PDMS layer: inlets 1.5 mm (D); perfusion channels: 200 × 100 µm (W × H) | SU-8          | UV photolithography | Glass         | O₂ plasma      | In-house         | CGG fabrication utilizing vertical channel gravity for flow styling |
| Ju, S.M. et al. [38]          | 2015 | PDMS          | 3      | Soft lithography | Gradient generator and cell culture microchamber channel: 100 µm (H); pneumatic channel H: 150 µm | SU-8          | UV photolithography | Glass         | O₂ plasma      | In-house         | Development of pump-free CGG with micropump system |
| Study                         | Year | Material     | Layers | Microfluidic Device                      | Mold                      | Device Assembly       | Manufacturing | New Technologies                                      |
|------------------------------|------|--------------|--------|------------------------------------------|---------------------------|-----------------------|--------------|-------------------------------------------------------|
| Pasirayi, G. et al. [39]     | 2014 | PDMS/PMMA    | 3      | Two PDMS layers 100 µm (T); control layer microchannels: 200 × 200 µm (W × H); CGG microchannels: 300 × 200 µm (W × H) | SU-8; UV photolithography | Glass; PDMS; PMMA | Clamping     | In-house; NR                                           |
| Li, E. et al. [40]           | 2014 | PDMS         | 1      | CGG mixers and 6 main channels: 300 × 100 µm (W × H); 18 cell chambers: 2.5 × 0.1 mm (D × H) | SU-8 and positive photoresist; UV photolithography | Glass; O₂ plasma; In-house | NR           | Integration of spheroid culture with CGG compared to 2D culture |
| Kwapiszewska, K. et al. [41] | 2014 | PDMS         | 1      | Microchamber: 50 µm (H); 18 microwells of 200 × 150 µm (D × H); microchannel: 200 µm (W) | PMMA; PDMS; CNC machining | PDMS; O₂ plasma; In-house | NR           | CCG array of hydrodynamic cell traps integration       |
| Fernandes, J.T.S. et al. [42]| 2014 | PDMS         | 1      | Serpentine-shaped microchannel: 50 × 9 µm (W × H) | SU-8; UV photolithography | Glass; PDMS; In-house | NR           | CCG array of hydrodynamic cell traps integration       |
| Jastrzebska, E. et al. [43]  | 2013 | PDMS         | 2      | Culture chamber: 1000 × 30 µm (D × H); microchannels: 100 × 50 µm (W × H) | Pro/Cap 50 and S1818; UV photolithography | Glass; O₂ plasma; In-house | NR           | CCG array of hydrodynamic cell traps integration       |
| Xu, Y. et al. [44]           | 2012 | PDMS         | 4      | Cell culture and the cytotoxicity assay, PDMS cavities (2 × 8 × 2 mm (H × L × W)); eight groups of impedance sensing electrodes (20 × 30 µm (W × interelectrode distance) | SU-8; UV photolithography | Glass; Thermal ball bonding; O₂ plasma; In-house | NR           | CCG array of hydrodynamic cell traps integration       |
| Yang, C.G. et al. [45]       | 2011 | PDMS         | NR     | Six circular channel 150 × 40 µm (W × H); serpentine channels 80 × 40 µm (W × H); inlet 0.3 mm (D); wedge-shaped chambers for cell culture 7 × 100 × 360 × 40 µm (L × W (narrow side) × W (wider side) × H) | AZ P4620; UV photolithography | NA; Air plasma; In-house | NR           | CCG array of hydrodynamic cell traps integration       |
| Study | Year | Material | Layers | Technology | Microfluidic Device | Mold | Device Assembly | Manufacturing | New Technologies |
|-------|------|----------|--------|------------|---------------------|------|----------------|---------------|-----------------|
| Jedrych, E. et al. [46] | 2011 | PDMS | 2 | Soft lithography | Matrix (5 × 5) of the culture microchambers 1000 × 30 µm (D × H) coupled with microchannels, creating the CGG; microchannels 100 × 50 µm (W × H) | Pro/Cap 50 and S1818 | UV photolithography | Glass | O₂ plasma | In-house | NR |
| Li, Y. et al. [47] | 2015 | PDMS | 2 | Soft lithography | Circular channels: 200 × 50 µm (W × H); serpentine branch channels: 200 × 50 µm (W × H); solution inlets: 1 mm (D); cylinder-shaped chambers (7 mm (D), 2.5 µm (H)) | Copper | CNC machining | Glass | O₂ plasma | In-house | CGG can generate one blank solution, seven mixture concentrations, and eight single concentrations for each metal solution |
| Yang, F. et al. [48] | 2011 | Glass | 2 | UV direct writing photolithography | CGG on top slide: microchannels 120 × 30 µm (W × H), connective channels 300 × 30 µm (W × H), seven embryo inlets 1.3 mm (D); sandwiched culture chambers 4 × 1.7 mm (D × T of glass plate) | NA | NA | Glass | Anodic bonding | In-house | Reusable glass chip with natural hydrophilicity |

**Abbreviations:** PDMS: polydimethylsiloxane; p82: polymer82; hw83: hydroxylated wax83; NA: not applicable; NR: not reported; OOC: organ-on-a-chip; CGG: concentration gradient generator; L: length; W: width; H: height; Vol: volume; D: diameter; T: thickness; SU-8: negative photoresist; S1813: positive photoresist; AZ P4620: positive photoresist; PMMA: poly(methyl methacrylate); CNC: computer numerical control; UV: ultraviolet; ITO glass: indium tin oxide glass; RTV: room-temperature vulcanizing; DLD: deterministic lateral displacement; µFSCD: microfluidic spheroid culture device.
Regarding microfluidic device fabrication, all evaluated devices were manufactured in-house, and the methods used in the studies are organized and described in this paragraph. Among the materials used, polydimethylsiloxane (PDMS) was predominant (91%) [16,17,19–27,29–44,46,47], and the device manufacturing technology used with this material was soft lithography, totaling 91% of the studies [16,17,19–27,29–44,46,47], being, in the studies that used microalgae [23–27], reported in 100% of the cases. Only 9% of the studies reported other technologies and materials [18,28,48], such as ultraviolet (UV) photolithography (3%) [48] in glass applied in the Zebrafish embryo model, silicon micromachining (3%) [28] with silicon in the tumor cells and other models groups, and 3D printing using a polymer as the main material mold, which was applied in one study from the microorganisms group [18].

Soft lithography methodology consists in pouring a polymer over a mold. The mold fabrication was performed mainly by UV photolithography (72.2%) [16,17,20,21,23–27,30,31,33–39,42–44,46,48], and 58.3% [16,17,20–27,31,33–39,41,42,44,46,48] of the materials used were negative photoresists, such as SU-8 and S1800, while only 5.5% [39,45] of studies utilized positive photoresists, such as AZ, all of these being from the tumor cells and other models group. Once again, only microalgae studies were unanimous on mold fabrication, using SU-8 material. A total of 15% of studies reported other mold manufacturing methods [19,22,29,41,47], with 12% reporting the use of computer numerical control (CNC), which was applied in different types of biological models [19,22,41,47], and 3% reporting the use of laser-based technology, which was applied in one study that used tumor cells and others as a model [29]. In 27.7% of studies [19,22,29,41,47], other materials were used, such as mold (glass, silicon, PDMS, poly(methyl methacrylate) (PMMA), copper, and Pro/Cap50), and only one study, which used a tumor human cell model, did not report this information [32].

Most of the devices (73%) were reported to have more than one sandwiched layer [16–19,22,23,25–27,29–39,43,44,46–48], 18% used structures in only one layer (two microalgae studies [20,24] and four cell studies [28,39,41,42]), 6% did not report the layers used [21,45], and in the study on the microdevice manufactured by 3D printing, this information was not applicable [18]. After the device’s completion, the channels were commonly sealed with a glass cover (62.8%) [16,17,21,23,25,28–33,35,37–39,42–44,46,48] or with polymeric sealing (31.4%) [19,20,22,24,26,27,34,36,39,41]. Only the studies with the zebrafish embryo model had unanimity in glass cover, and in 5.8% [18,45], this was not applicable. The bonding techniques used to cover the microdevices were, mainly, plasma bonding (63.6%) [16,20,22,23,25,27,29–32,34,35,37–39,41,43,45,47], followed by uncured PDMS (12.1%) [17,19,26,42] and anodically bonding (6%) [28,48]. A few studies reported other techniques (6%) [36,39,44], and in others, this information was not applicable or reported (12.3%) [18,21,24,33]. The sealing techniques were evenly split between all four groups of the biological model.

New technologies analysis showed innovations in their fabrication or materials, such as the development of facilitators in relation to the main reported problems in microfluidics, such as shear and bubbles (27%) [16,17,21,27,35,37,38,42,44], cost reduction, and rapid prototyping (12%) [18,28,36,48], and integration of other systems (9%) [19,20,24], such as electrodes, for example. Another 24% [22,23,29,31,32,34,41,47] presented precise technologies for the analysis of the organism in question, enabling customized development. A total of 27% of articles did not report new technologies [25,26,30,33,39,43,45,46]. Finally, regarding the dimensions of microfluidic devices used in the selected studies, mainly in the culture chamber and channel parts, the measurements were very particular for the purpose of the study; the larger chambers used a higher concentration of microorganisms in cultures or 3D culture. The devices made for the microalgae model had measurements with less variation, and, for the zebrafish embryos model, the chamber height was much shorter than for the others. According to the design and the structures of the microdevice developed by each author, some of the CGG’s particularities are exemplified in Figure 2.
Figure 2. Schematic diagram of microfluidic devices with CGG system for toxicological analysis, representing some of the studies selected in this systematic review. (A) Representation of device layers, gradient generator structure, details of fluid mixing units and air bubble valves. Adapted with permission from [44], Biosensors and Bioelectronics. (B) Project showing a physical map of the CGG system and the photomicrograph of Pyramimonas sp. and Chlorella. Adapted with permission from [23], Sensors (Switzerland). (C) A schematic showing the design of a µFSCD with a concentration gradient generator. It exposed the structures, dimensions, and characteristics of the two layers, adapted with permission from [34], Molecules. (D) Construction of the Sphero Chip system proving the measurement principle of the experimental scheme and results of the computational modeling of a CGG structure. Adapted with permission from [41], Lab on a Chip. (E) The microdevice contains eight sets of C-Chambers, which can simultaneously enable eight sets of noninterfering ASTs with each other. Antibiotics can be preincorporated into the C chambers with a specific mass gradient. AST and MIC results can be obtained by comparing the fluorescence intensities between each set of C-Chambers. Adapted with permission from [16], Biosensors and Bioelectronics. (F) CGG microdevice used for toxicity tests based on marine phytoplankton motility containing four units connected to a central removable outlet. Shown is the enlarged image of the single-frame unit containing an upstream CGG and downstream diffusible cameras. Motility signals can be collected in real time. Adapted with permission from [25], Marine Pollution Bulletin. (G) Schematic design of the CGG microfluidic chip with cell chambers (top panel) and the chip manufactured with pumping machine (bottom panel). Chamber-diffused Rh-123 (green) and morphological characteristics of A549 cells
with or without CAF matrix are shown. Adapted with permission from [37], *PLoS ONE*. (H) CGG containing four parallel operational modules including inputs CSE: 18 parallel cell chambers and 6 cell inputs. A CSE concentration gradient is shown from entry one to six, adapted with permission from [40], *Journal of Thoracic Oncology*. (I) Schematic overview of the microfluidic device with a CGG and chambers with passive hydrodynamic cell trap arrays. It shows details of branching and diffusional mixing of two fluorescent fluids with different concentrations and optical micrograph of cell traps in PDMS. Adapted with permission from [42], *Lab on a Chip*. (J) Schematic drawing of the CGG device, illustrating cross-section and theoretical profiles of Ciprofloxacin concentration in the observation channel. Antibiotic solutions with $3 \times$ MIC (blue curve) or $6 \times$ MIC (orange curve). Adapted with permission from [17], *Frontiers in Microbiology*. **Abbreviations:** CGG: concentration gradient generator; µFSCD: microfluidic spheroid culture device; ASTs: antibiotic susceptibility tests; Rh−123: Rhodamine; A549: adenocarcinoma human alveolar basal epithelial cell line; CAF: cancer-associated fibroblasts; CSE: cigarette smoke extracts; PDMS: polydimethylsiloxane; MIC: minimal inhibitory concentration.

### 3.3. Concentration Gradient Generator Characteristics of Microfluidic Device

For the development of microfluidic devices capable of generating concentration gradients, it is necessary to establish general and functional characteristics, such as, as analyzed in Table 2, the methods and types of systems used to generate gradients, number of concentrations generated, linearity of the gradient, variation in concentrations of the compounds evaluated, and time to achieve gradient stability, as well as the duration of stability, in addition to information on simulation methods, validation, and advantages. Most studies used the Christmas tree gradient generation system (Figure 2A–D,F,G,I) [21–27,29,30,34–39,42–48], which uses the convective method, either associated or not associated with other systems or with certain modifications. Less frequently, other systems also used convective methods, such as T-shaped channels (6%) (Figure 2G) [20,37], serpentine channels (Figure 2A–D,F–I) [23,25,34,37,40–42,44] cascade mixing (Figure 2H) [40], and 3D microchannel networks [18] (3% each). Diffusion methods used to generate gradients were associated with Y-junction systems (6%) [21,28], the snake model [24], droplet generation (Figure 2E) [16], static-pressure-driven CGG [31], and membrane systems (Figure 2J) [17], with 3% each.
| Study          | CGG Design                  | Concentrations Details | Advantages                                                                 |
|---------------|-----------------------------|------------------------|-----------------------------------------------------------------------------|
| Zeng, W. et al. [16] | Diffusion Droplet generation | Eight C-Chamber sets, which can simultaneously enable eight AST sets without interfering with one another Two trapezoid reservoirs in the upper layer and a rectangular reservoir in the bottom layer, with a porous membrane in between | Uses the properties of diffusion of gases by vacuum pressure levels for drug gradient formation |
| Nagy, K. et al. [17] | Diffusion Membrane          | Two trapezoid reservoirs in the upper layer and a rectangular reservoir in the bottom layer, with a porous membrane in between A tetrahedrally arranged network of nodal microchannel units, geometrically symmetric in 3D space and capable of generating three inherently symmetric fluid gradients | No shearing effect thanks to flow-free diffusion generation gradient |
| Sweet, E. et al. [18] | Convective 3D microchannel network | In two layers, at low spinning speed, with the help of centrifugal force, the fluidic content supplied by the source chamber will flow along the spiral channel and fill the metering chambers, while the redundant fluid will flow into the waste chamber Eight T-shaped channels and eight ellipsoid observing chambers. Each T-shaped loading channel has been connected with a chamber and two separate inlets | Integration of tetrahedrally arranged nodal combination–mixing–splitting units with a vertical u-mixing to obtain shearing-free and linear concentration flow |
| Tang, M. et al. [19] | NR Centrifugal CGG          | 16 Linear AMP (0-8 µg/mL; increases of 0.5 µg in each concentration) Dye solutions | Generates 16 accurate concentration levels, with slight variations, with the use of centrifugal force |
| Zhang, B. et al. [20] | Convective and diffusive Serpentine channels and T-shaped channel | 8 Linear 0, 20, 50, 80, 100, 80, 50, + 20 µM (substance mixing) FITC |                                                             |
### Table 2. Cont.

| Study | CGG Design | Concentrations Details | Method of Generation | Gradient System | Structure | Concentration Numbers | Concentration Type | Range | Validation | Stable Gradient Formation Time (sec) | Flow Simulation | Stable Gradient Duration (min) | Advantages |
|-------|------------|------------------------|----------------------|-----------------|-----------|-----------------------|-------------------|-------|------------|------------------------------------|----------------|-----------------------------|------------|
| DiCicco, M. and Neethirajan S. [21] | Convective and diffusive | Christmas tree and Y-junction Microdevice consists of two solution inlets, a Christmas-tree-shaped CGG, one bacteria inlet, a straight main channel, and one outlet Radial worm dispenser with 32 trap-construction chambers and 4 multiple-gradient generators with a regular Christmas tree shape | 9 | Linear | NR | FITC | NR | NR | NR | NR | NR | NR |
| Yang, J. et al. [22] | Convective | Christmas tree | Radial worm dispenser with 32 trap-construction chambers and 4 multiple-gradient generators with a regular Christmas tree shape | 8 | Linear | 0, 14.3, 28.6, 42.8, 57.1, 71.4, 85.7, and 100 mM (substance mixing) | FITC | NR | NR | NR | NR | NR |
| Wang, Y. et al. [23] | Convective | Christmas tree CGG with two inlets and a rectangular cross-section The snake geometry is created by interactively folding a channel in an equal interval until the design specification is met according to the rules: L = (N + 1)ws + Ls − 3w, where w is the channel width, ws is the snake width, Ls is the snake length, N is the number of snake’s bends, and D is the snake density (Ls/N) | 6 | Linear | NaClO (250 ppm) (0, 50, 100, 150, 200, and 250 ppm); NaClO (500 ppm) (0, 100, 200, 300, 400, and 500 ppm) | NR | NR | NR | NR | NR |
| Han, B. et al. [24] | Diffusion | Snake model | Cu (0.8, 1.6, 2.4, 3.2, 4 µM); Hg (0.8, 1.6, 2.4, 3.2, 4 µM); Zn (24, 48, 72, 96, 120 µM); Cd (16.5, 33, 49.5, 66, 82.5 µM) | 5 | Linear | NR | CFD-ACE simulations | NR | Combination of linear channels with single-cell snake mixers to minimize design effort |
| Study | CGG Design | Concentrations Details |
|-------|------------|-------------------------|
|       | Method of Generation | Gradient System | Structure | Concentration Numbers | Concentration Type | Validation | Stable Gradient Formation Time (sec) | Flow Simulation | Stable Gradient Duration (min) | Advantages |
| Zheng, G. et al. [25] | Convective | Christmas tree | Four uniform structure units connected by a central outlet, each containing an upstream CGG with two inputs and downstream diffusible chambers | 8 | Linear | Hg (0, 0.43, 0.85, 1.28, 1.71, 2.13, 2.56, and 3.0 µM); Pb (0, 1.62, 3.24, 4.86, 6.48, 8.10, 9.72, and 11.34 µM); Cu (0, 0.625, 1.25, 1.875, 2.5, 3.125, 3.75, and 4.375 µM); Phenol (0, 1.29, 2.57, 3.86, 5.14, 6.43, 7.71, and 9.0 mmol/L); Phenol + Cu (0, 0.325, 0.65, 0.975, 1.3, 1.625, 1.95, and 2.275 mmol/L) | Rh | NR | NR | 25 | Prevents any active flow through the chambers and possible disruption of cell position, movement, or intercellular interaction |
| Zheng, G. et al. [26] | Convective | Christmas tree | Upstream serpentine channels and downstream parallel diffusion channels. Sixteen isolated dead-end chambers for microalgal culture and imaging | 8 | Linear | 0, 1C/7, 2C/7, 3C/7, 4C/7, 5C/7, 6C/7 (each initial concentration—C) | Rh | 320 | NR | 50 | A torque-actuated valve system without use of an external power or pressure source |
| Zheng, G. et al. [27] | Convective | Christmas tree | Has a regular Christmas tree CGG shape that results in 8 gradients integrated with chemostatic chambers for microalgal culture | 8 | Linear | NR | Rh | 180 | NR | 20 | Use of different height than CGG flow channels of culture chambers to obtain no-return flow |
| Chennampally, P. et al. [28] | Diffusion | Y-junction | The overall geometry is designed to mimic the primary aspects of the diffusion-based patterning of the neural tube | 11 | Linear | Rapamycin (0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8, and 2.0 µM) | FITC | Established in <1800 | COMSOL | Maintained indefinitely | Generates a gradient within the chamber that corresponds with SHH diffusion profiles to mimic neural tube |
### Table 2. Cont.

| Study | CGG Design | Concentrations Details |
|-------|------------|------------------------|
| Method of Generation | Gradient System | Structure | Concentration Numbers | Concentration Type | Range | Validation | Flow Simulation | Stable Gradient Duration (min) | Advantages |
| Yin, L. et al. [29] | Convective | Christmas tree | Christmas tree with two inlets | 5 | Linear | CDDP + CsA (0, 10, 20, 30, and 40 µmol/L); CDDP + Cim (0, 20, 40, 60, 80 µmol/L); GM (0, 10, 20, 30, and 40 mmol/L) | Dye solutions | NR | NR | NR | NR |
| Jaberi, A. et al. [30] | Convective | Christmas tree and micropillars | Microchambers (cell/drug) placed after each serpentine channel of the Christmas tree design. In another design, micropillars were also built into chambers to produce a gradient within the chambers | 6 | Linear | DOX (0, 6, 12, 18, 24, and 30 µg/mL) | DOX | NR | COMSOL | NR | Micropillars on each microculture chamber to produce a gradient within the chambers |
| Zhao, X. et al. [31] | Diffusion | Static-pressure-driven CGG | Consists of two rows, with seven inlets each, and eight mixing chambers. The top row of inlets is connected to the last seven channels, while the bottom row is connected to the first seven channels | 8 | Linear | H₂O₂ (25 to 175 µM/500 µM (lethal dose)) | FITC | NR | COMSOL | NR | Pump-free CGG generates a shear-free microenvironment with a tunable network to generate predefined biochemical gradients |
| Qin, Y. X. et al. [32] | NR | NR | Consists of 6 main channels and 18 cell chambers. The CGG module included five cascaded-mixing stages | 6 | NR | Theoretical proportion 0:1:3:5:7:9 | NR | NR | NR | NR | NR |
| Luo, Y. et al. [33] | NR | Circular concentration gradient | Circular CGG with radial splitting-mixing—splitting-mixing processes | 5 | NR | 0, 3C/4, C/2, 4C/3 and C (each initial concentration—C) | SF | NR | NR | NR | Radial splitting-mixing integration with a serpentine channel to obtain shearing-free and linear concentration |
| Lim, W. and S. Park [34] | Convective | Christmas tree | Christmas tree with two inlets and connected to a culture array | 5 | Linear | Irinotecan (0, 1.25, 2.5, 3.75 and 5 µM) | FITC | NR | NR | NR | NR |
Table 2. Cont.

| Study            | CGG Design                           | Concentrations Details                                                                 |
|------------------|--------------------------------------|----------------------------------------------------------------------------------------|
|                  | Method of Generation | Gradient System | Structure | Concentration Numbers | Concentration Type | Range                          | Validation | Stable Gradient Formation Time (sec) | Flow Simulation | Stable Gradient Duration (min) | Advantages |
| Jin, D. et al. [35] | Convective                | Christmas tree | The top PDMS layer with two drug inlets integrated with six downstream 2D cell culture channels terminating at HUVEC inlets. The bottom PDMS layer has six 3D cell culture units | 6                      | Linear                          | PTX (0.01–0.49 µg/mL); CDDP (0.09–4.95 µg/mL); 5-FU (2.3–390 µg/mL) | Rh         | NR                               | NR                | NR                             | NR         |
| Hong, B. et al. [36] | Convective                | Christmas tree | A regular Christmas tree shape within S-shaped mixers on two inlets and five outlets | 5                      | Linear                          | DOX (4, 41, 90, 143, and 182.5 µg/mL) | Dye solutions | 900                               | NR                | NR                             | NR         |
| Ying, L. et al. [37] | Convective and diffusive | Christmas tree and T-shaped channel | A combination of a linear CGG with two inlets and four downstream parallel cell culture units with two oval-shaped modules | 4                      | Linear                          | PTX (0, 1.28, 2.99, and 4 µM) | Rh         | 1800                              | NR                | NR                             | NR         |
| Ju, S. M. et al. [38] | Convective                | Christmas tree | Upstream CGG with six-step serpentine array to generate a diverse gradient at each step from two stock solutions | 8                      | Linear                          | APAP (0, 5.7, 11.4, 17.1, 22.8, 28.5, 34.2, or 40 mM) | Dye solutions and FITC | NR                               | NR                | NR                             | NR         |
| Pasirayi, G. et al. [39] | Convective                | Christmas tree | Two inputs connecting the regular Christmas tree shape with six outputs interconnected with four gradient culture microchambers, which have separate inlet and outlet reservoirs | 6                      | Linear                          | PCN (0, 20, 40, 60, 80, and 100 mM); PTX + Aspirin (0, 2, 4, 6, 8, and 10 mM) | Dye solutions | NR                               | NR                | NR                             | NR         |
| Li, E. et al. [40] | Convective                | Cascaded mixing | Consists of four parallel operating modules for simultaneous culture of four cell samples, and each module includes a CGG, 6 main channels, and 18 cell chambers | 6                      | Linear                          | CSE (0, 2.37, 12.28, 19.86, 46.79, and 91.88%) | NR         | NR                               | NR                | NR                             | NR         | Cascaded-mixing stages that generated linear concentrations by adjusting the flow rate of two merging solutions in each stage through controlling channel length proportional to fluidic resistance |
| Study | Method of Generation | CGG Design | Concentrations Details | Flow Simulation | Stable Gradient Duration (min) | Advantages |
|-------|----------------------|------------|------------------------|----------------|-------------------------------|------------|
| Kwapiszewska, K. et al. [41] | Convective | Serpentine channels | Spheroid culture microchambers were placed in an array of three serpentine channels, each containing four microculture chambers | 3 Linear | 5-FU (0, 0.125, 0.5, and 1 mM) NR Less than 20 COMSOL NR NR |
| Fernandes, J. T. S. et al. [42] | Convective | Christmas tree | Nine chamber sets, each containing hydrodynamic traps for yeast cells, and a chemical gradient generator has three inlets (solution inlets) that allow the insertion of chemical solutions of different compositions | 9 Linear | Ascorbic acid (0, 0.13, 0.25, 0.38, 0.50, 0.63, 0.75, 0.88, and 1% initial concentration percentage) | FITC 1 NR NR NR |
| Jastrzebska, E. et al. [43] | Convective | Christmas tree | A regular Christmas tree CGG shape that results in five gradients integrated on five meander modules each, totaling 25 culture microchambers | 5 Linear | 24 hrs: Celbx (39–83 µM) and 5-FU (93–202 µM); 48 hrs: Celbx (19–117 µM) and 5-FU (8–253 µM) | FITC NR NR NR NR | Fully reusable; i.e., it can be used several times for various cell culture and cytotoxic experiments |
| Xu, Y. et al. [44] | Convective | Christmas tree | The device contained an upstream CGG with a regular serpentine mixer, eight air bubble valves, and downstream parallel cell culture chambers, aligned with the bottom cavities | 8 Linear | NR NR NR NR NR | Fluid mixing units on CGG channels |
| Yang, C. G. et al. [45] | Convective | Christmas tree | Radial channel composed of multicircle channels and parallel branch channels. Latitudinal, six circular channels are arranged concentrically. Longitudinally, the serpentine branch channels are arranged symmetrically around each of the circular channels | 65 Linear | 5-FU (0–600 mg/mL); CDDP (0–400 mg/mL) and 5-FU + CDDP (0–600 mg/mL) | Rh 30 NR NR | Integration of circular channels and serpentine branch channels to generate more concentration than conventional method |
| Study            | CGG Design                | Concentrations Details |     |     |     | Flow Simulation | Stable Gradient Duration (min) | Advantages                                      |
|------------------|----------------------------|-----------------------|-----|-----|-----|-----------------|---------------------------------|------------------------------------------------|
|                  | Method of Generation | Gradient System | Structure | Concentration Numbers | Concentration Type | Range | Validation | Stable Gradient Formation Time (sec) |                             |                              |
| Jedrych, E. et al. [46] | Convective             | Christmas tree       | Consists of a matrix (5 × 5) of culture microchambers coupled with microchannels creating the CGG, which includes two inlets and five outlets | 5 | Linear | 5-FU (0, 75, 150, 225, and 300 µM) | NR | NR | NR | NR | NR |
| Li, Y. et al. [47] | Convective             | Christmas tree       | Composed of multicircle channels and parallel branch channels, latitudinally, three circular channels are arranged centrically and longitudinally, the serpentine branch channels; three inlets are located inside the first level, and a cylinder-shaped chamber array is located downstream of the branch channels in the outermost level | 24 (8 gradients per drug) | Linear | Eosin Y, FITC, and ethanol | NR | NR | NR | NR | NR |
| Yang, F. et al. [48] | Convective             | Christmas tree       | Contains a simple Christmas tree CGG with two inlets generating seven concentration gradients | 7 | Linear | Theoretical proportion 0, 16.7, 33.3, 50, 66.7, 88.3, 100 µg/mL | NR | 96 | NR | NR | NR |

**Zebralish embryos**

Centripetal geometry and the arrangement of concentric serpentine channels are able to generate mixing and single concentrations automatically.

Abbreviations: NR: not reported; CGG: concentration gradient generator; AST: antibiotic susceptibility testing; HUVEC: human umbilical vein endothelial cell; PDMS: Polydimethylsiloxane; KAN: Kanamycin; AMP: Ampicillin; TAC: Tetracycline; CIPRO: Ciprofloxacin; MIC: minimal inhibitory concentration; AMK: Amikacin; µM: micromolar; mM: millimolar; ppm: parts per million; CDDP: Cisplatin; CsA: Cyclosporin A; Cim: Cimetidine; GM: Gentamycin; DOX: Doxorubicin; PTX: Paclitaxel; 5-FU: 5-Fluorouracil; APAP: Acetaminophen; PCN: Pyocyanin; CSE: cigarette smoke extracts; Celbx: Celecoxib; DI water: deionized water; FITC: fluorescein isothiocyanate; Rh: Rhodamine; SF: sodium fluorescein; PBS: phosphate-buffered saline; CDF-ACE: computational fluid dynamics; SHH: sonic hedgehog; ECIS: electric cell–substrate impedance sensing.
Interestingly, two of the studies, one from the microorganisms group and another from the tumor cells group, used a combination of convective and diffusive methods, such as serpentine/T-shaped channels [20], and Christmas tree/Y junction (Figure 2G) [37]. Only three studies did not report the generation method used, and the systems used were centrifugal CGG [19] and circular concentration gradient [33], while the study by Qin, Y.X. et al. reported neither the method nor the system [32]. The CGG structure was designed according to the gradient system used and the generation method, to ensure efficiency in toxicity screening. These important aspects of the CGG structure are highlighted in Table 2.

An alternative way to evaluate the functionality of the structures and the efficiency of the generation of gradients is the use of software to simulate the flow. In this review, only 18% of studies used COMSOL (software for multiphysics simulation) [17,18,28,30,31,41], mainly those on tumor cells and other models and microorganisms. The study by Han, B. et al. (microalgae group) performed the simulation through computational fluid dynamics (CFD) [24]. In the studies with embryos, simulation was not reported [47,48].

The devices developed in the selected studies generated from 2 to 65 different concentrations, with the greatest variation observed in the tumor cells and other models group, at 3 to 65 concentrations generated, and the lowest in the microalgae group, at 5 to 8 concentrations, with the microorganism and the zebrafish embryo groups having produced from 5 to 8 and 2 to 24 concentrations, respectively. The generated concentration values were reported in 91% of the studies, and they were considered linear [16,17,19–31,34–48].

For the creation of the perfect gradient, a certain amount of time is required, and only 30% of the studies reported these data, varying from 1 to 1800 s [16,26–28,36,37,41,42,45,48]. The stability time, also an important parameter, was reported in only 12% of studies [25–27], varying between 20 and 50 min in the microalgae group, with one study from the tumor cells and others group reporting an indefinite time of maintenance [28], while the other groups did not report this parameter.

One of the final steps in the development of the devices, validation, which aims to ensure the correct functioning of the gradient generator system, was reported in 55% of studies, with the main method used for this purpose being fluorescent agents [18,20–22,25–28,31,33–35,37,38,42,43,45,47], followed by food coloring, used in 12% of studies, one from the microorganism group [19] and three from the cells group [29,36,40]. Only one study (3%) used Doxorubicin (DOX) for validation [30]; the other 30% did not report this step [16,17,23,24,32,40,41,44,48].

Regarding the concentrations of drugs or stimuli used in the study of toxicity, some of the more frequently used substances showed a similar pattern. For the antibiotic toxicity screening in microorganisms, the most frequently used antibiotics were Ampicillin (AMP) and Tetracycline (TAC), with concentrations ranging from 0 to 13.1 µg/mL [16,18,19], while the concentration of Ciprofloxacin (CIPRO) ranged from 0 to 96 µg/mL [17]. For the evaluation of the toxicity in microalgae, the range of concentrations of copper (II) sulfate varied from 0 to 4.375 µM, and for mercury (II) chloride, from 0 to 4 µM [24,25]. The concentration variation in the main chemotherapies applied in toxicity screening in the tumor cells group varied from 0 to 600 mg/mL for 5-Fluorouracil (5-FU) [41,43,45,46], from 0 to 400 mg/mL for Cisplatin (CDDP) [29,35,45], 0 to 3.4 mg/mL for Paclitaxel (PTX) [35,37], and from 0 to 0.01825 mg/mL for DOX [30,36]. For studies related to embryogenesis, a lower range was used (0–100 µg/mL) for Adriamycin (ADM), DOX, 5-FU, and CDDP [48].

Some advantages regarding the CGG system and microdevice structure were reported in 55% of the studies. Shear-free fluid flow was a concern considered by 32% of the works, where shear minimization was provided, mainly, by the shape of the mixing channels, difference in heights in relation to the culture chamber, and use of splitting–mixing systems associated with serpentine channels [18,19,28,30,36,38]. Automation was also considered by 26% of the studies, so vacuum pressure channels (Figure 2A), centrifugal force [19], snake-channel torque-operated valves [26], and centripetal geometry [47] were used to minimize handling and optimize the generation of gradients [19,24,26,44,47]. To guarantee the linearity of the gradients, 21% of the studies reported the optimization of the structures by
modifying the length of the channels (Figure 2A,H) [40,44], using micropillars in the culture chambers [30], radial splitting–mixing integration with a serpentine channel [33,45], and cascaded mixing (Figure 2H) [23,30,33,40]. In addition, 11% reported a concern regarding the high performance of these devices, the number of concentrations generated using radial splitting–mixing integration with a serpentine channel, and centripetal geometry together with the arrangement of concentric serpentine channels [45,47]. Only 5% of the studies reported a concern with mimicking the gradient in vivo [28] and reusing the developed devices [43].

3.4. Biological Model Used for Toxicity Evaluation in the CGG Microfluidic Device

Table 3 shows the details of the biological model used, the characteristics of the culture environment, and the toxicity conditions analyzed. The main microorganisms used as biological models were, firstly, bacteria (71.4%), with the great majority of the studies choosing different *E. coli* strains [16–19]—with exception of the study by DiCicco [21], in which a canine bacteria species (*S. pseudintermedius*) was used—and, secondly, *Caenorhabditis elegans* (*C. elegans*) nematode (28.6%) [20,22]. Both models were utilized for antibiotic toxicity screening—with exception of the study by Zhang B [20], which employed manganese chloride combined with vitamin E, resveratrol, and other substances. The most tested antibiotics were AMP [16,19], CIPRO [17,18], and TAC [16,18], followed by Kanamycin (KAN) [16], Amikacin (AMK) [18], Fosfomycin (FO) [21], and Amoxicillin (AMX) [22], with an incubation time between 4 and 72 h. The longer periods of incubation were associated with the evaluation of genetic mutation and antibiotic resistance. The drugs’ flow rates were reported in less than half of the studies (42.9%) [20–22], with a range of 10 to 300 µL/h, and the organisms were mostly cultured intra-CGG (85.7%) [16,17,19–22] and in 2D culture, with only two studies reporting the use of 3D culture (28.6%) [16,17], one being a co-culture. The average number of organisms for the studies that used bacteria was around 10⁶ cfu/mL (10⁸ for canine bacterium) and, for those based on nematodes, 1 worm/mL. Regarding the culture environment, the principal medium employed for bacteria culture was Luria–Bertani broth (for the *E. coli* strains), excluding the study based on *S. pseudintermedius*, which used Columbia agar associated with Tryptic soy broth plus glucose, and for *C. elegans*, a nematode growth medium was chosen. All bacteria were incubated with temperatures ranging from 30 to 37 °C, and the nematode studies applied lower temperatures around 20 to 25 °C.
Table 3. Biological model used for toxicity evaluation in CGG microfluidic device.

| Study                  | Biological Model | Culture Environment | Toxicity Conditions | Flow Rate; Diffusion Constant | Incubation Time (h) |
|------------------------|------------------|---------------------|---------------------|-----------------------------|---------------------|
| **Microorganism**      |                  |                     |                     |                             |                     |
| Zeng, W. et al. [16]   | Bacterium E. coli | E. coli k12 NR Intra-CGG 3D culture | LB broth medium | AMP (100 mg/mL; MIC: NR), KAN (10 mg/mL; MIC: 7.1 µg/mL), TAC (10 mg/mL; MIC: ~3 µg/mL) + DI water | NR; NR 8 |
|                        |                  | E. coli K-12 Alamar Blue (1 µg/mL) and LB broth medium (1:10) |                     |                             |                     |
| Nagy, K. et al. [17]   | Bacterium E. coli | W3110-GFP NR Intra-CGG 3D Co-culture (1:1) | LB broth medium and antibiotic-free LB | CIPRO (3 and 6 × MIC) | NR; 6.87 × 10⁻⁶ cm²/s 48 and 72 |
| Sweet, E. et al. [18]  | Bacterium E. coli | BL21-DE3 (2) Agilent Technologies, CA, USA Extra CGG 2D culture | LB broth medium | TAC, CIPRO, and AMK; buffer (control) combined each other | NR; NR NR |
| Tang, M. et al. [19]   | Bacterium E. coli | BL21-DE3 DBE-CEAS-Nanjing University, China Intra-CGG 2D culture | LB medium + KAN (50 µg/mL) | AMP | NR; NR 5 |
| Zhang, B. et al. [20]  | Nematode C. elegans | BZ555 (5) NR Intra-CGG 2D culture | NCM with OP50 Dark 20 | MnCl₂ (100 mM) + vitamin E, resveratrol, or quercetin (100 µM), and K solution | 5 µL/min; NR 48 |
| Dicicco, M. and Neethirajan S. [21] | Canine bacterium S. pseudintermedius | MRSP A12 University of Guelph, Ontario Veterinary College, Canada Intra-CGG 2D culture | Columbia agar; TSB-G tubes at a 0.5 McFarland standard | FO (16, 32, and 64 µg/mL) | 10 µL/h; NR 24 |
| Study | Biological Model | Culture Environment | Toxicity Conditions |
|-------|-----------------|---------------------|---------------------|
|       | Origin          | Type                | Organism            | Source | Biological Structure | Number of Organisms | Culture Medium | Temperature (°C) | Condition Environment | Stimulus/Drug | Flow Rate; Diffusion Constant | Incubation Time (h) |
|       |                 |                     |                     |        | 2D culture           | 1–1.5 worms/µL      | S. Aureus; 10% BHI–M9 medium; 5 µg/mL nalidixic acid E. coli: op90; NGM + 5 µg/mL nalidixic acid S. Aureus; 10% BHI–M9 medium | 25 | NR | AMX   | 10 µL/min; NR | for 12, 24, 36, 48, and 60 |
| Yang, J. et al. [22] | Nematode | C. elegans | Glp-4 (bn2ts)sek-1 (km4) | NR | Intra-CGG | 2D culture | S. Aureus; 10% BHI–M9 medium; 5 µg/mL nalidixic acid E. coli: op90; NGM + 5 µg/mL nalidixic acid S. Aureus; 10% BHI–M9 medium | 25 | NR | AMX, aloe-emodin, rhein, and emodin with DMSO at 2% | 48 |
| Wang, Y. et al. [23] | Green microalgae (Chlorophyta) | Marine microalgae | Pyramimonas sp. (chl-1) | LOPSR, Dalian, China | Intra-CGG | 2D culture | 240 cells/µL; 580 cells/µL | Enriched seawater medium | 22-25 | NR | NaClO (250 ppm) NaClO (500 ppm) CuSO₄·5H₂O; HgCl₂, CdCl₂, 2.5H₂O, ZnSO₄·7H₂O; single and binary mixing | 12 |
| Han, B. et al. [24] | Green microalgae (Chlorophyta) | Marine microalgae | P. subcordiformis (chl-6) | KLMB, IOCAS, CAS, China | Intra-CGG | 2D culture | >10¹⁰ | F/2 medium | ~25 | 60 µmol photon m²/s | 1.5 µL/min; NR | 1 |
| Zheng, G. et al. [25] | Green microalgae (Chlorophyta) | Marine microalgae | P. subcordiformis (chl-6) | Chinese coast | Intra-CGG | 2D culture | 10⁸ individuals/mL | F/2 medium | 25 ± 0.5 | 60 µmol photon m²/s | CuSO₄·5H₂O (5 µmol/L); Pb(CH₃COO)₂·3H₂O (11.34 µmol/L); HgCl₂ (4.4 µmol/L) and phenol (9 mmol/L) CuSO₄·5H₂O (4.34 µmol/L); Pb(CH₃COO)₂·3H₂O (13.3 µmol/L); HgCl₂ (10 µmol/L) and phenol (12 mmol/L) | 50 µL/min; NR | 2 |
Table 3. Cont.

| Study | Biological Model | Origin | Type | Organism | Source | Culture Site | Biological Structure | Number of Organisms | Culture Environment | Temperature (°C) | Condition Environment | Stimulus/Drug (mM) | Flow Rate; Diffusion Constant | Incubation Time (h) |
|-------|-----------------|--------|------|----------|--------|--------------|---------------------|---------------------|---------------------|----------------|------------------------|----------------|--------------------------|------------------|
| Zheng, G. et al. [26] | Green microalgae (Chlorophyta) | Marine microalgae | P. subcordiformis (chl-6) | Extra CGG | 2D culture | >10³ | F/2 medium + CuSO₄·5H₂O | 25 | CO₂/O₂; 80 µmol photon/m²/s | 1 µL/min; 6 × 10⁻⁶ cm²/s | 1 µL/min; 6 × 10⁻³ cm²/s | CuSO₄·5H₂O (0-25 µmol/L) | 72 | |
| | Diatom (Bacillariophyta) | Red microalgae (Rhodophyta) | P. helgolandica var. tsingtaoensis (chl-5) | | | | F/2 medium + CuSO₄·5H₂O | | CuSO₄·5H₂O (0-10 µmol/L) | | CuSO₄·5H₂O (0-23 µmol/L) | | |
| Zheng, G. et al. [27] | Green microalgae (Chlorophyta) | Marine microalgae | P. subcordiformis (chl-6) | Intra-CGG | 2D culture | 10⁶ individuals/µL | F/2 medium + CuSO₄·5H₂O and CdCl₂·2.5H₂O | 25 | 60 µmol photon/m²/s | 0.1 µL/min; 6 × 10⁻³ cm²/s | 1.5 | |
| | | | P. helgolandica var. tsingtaoensis (chl-5) | | | | | | | | | CuSO₄·5H₂O (0-23 µmol/L); CdCl₂·2.5H₂O (225 µmol/L); single and Cu and phenol mixture | | |

Tumor cells and other models

| Study | Biological Model | Origin | Type | Organism | Source | Culture Site | Biological Structure | Number of Organisms | Culture Environment | Temperature (°C) | Condition Environment | Stimulus/Drug (mM) | Flow Rate; Diffusion Constant | Incubation Time (h) |
|-------|-----------------|--------|------|----------|--------|--------------|---------------------|---------------------|---------------------|----------------|------------------------|----------------|--------------------------|------------------|
| Chennampally, P. et al. [28] | Mice [30] | Embryonic stem cell | ESC-WT A315T | Primary cell | Intra-CGG | 2D and 3D culture | 10⁶–10⁷ | Fresh medium, Geltrex | 37 | 5% CO₂ | Rapamycin (1 µM) | ~100 µL/hr; 4.9 × 10⁻⁵ cm²/s | 168 | |
| Yin, L. et al. [29] | Human | Renal proximal tubule epithelial cells | RPTCs | Primary cell | Intra-CGG | 3D co-culture | 5 × 10⁶ | High-glucose DMEM; ECM + collagen | 37 | 5% CO₂ | CDDP, GM, CsA, and Cim | 10–100 µL/min; NR | 168 | |
| Jaberi, A. et al. [30] | Human | Epidermoid carcinoma | A431-DPNTP A431-S2849GDP | Prof. Kathleen Green, NU; Prof. James K. Wahl, UNMC | Intra-CGG | 3D co-culture | 10⁶ | GelMA; DMEM + FBS (10%) + P-S (1%) | NR | NR | DOX 98-102% | 0.1 µL/min (bottom-top) and 0.2 µL/min (end to middle); NR | 24 | |
| Study | Origin | Type | Organism | Source | Culture Site | Biological Structure | Number of Organisms | Culture Medium | Temperature (°C) | Condition Environment | Stimulus/Drug  (11) | Flow Rate; Diffusion Constant | Incubation Time (h) |
|-------|--------|------|----------|--------|--------------|----------------------|---------------------|----------------|----------------|----------------------|----------------------|--------------------------|---------------------|
| Zhao, X. et al. [31] | Mouse NIH/Swiss embryo | Fibroblast cell | NIH 3T3 | NR | Intra-CGG | 2D culture | $3.4 \times 10^3$ | DMEM medium + FBS (10%) | 37 | 5% CO₂ | Low and lethal dose of H₂O₂ | 0.2 mL/s; $4.9 \times 10^{-10}$ m²/s | 120 |
| Qin, Y. X. et al. [32] | Human | Bronchial epithelial cells | 16HBE | SPF-EAC-DMU, China | Intra-CGG | 2D culture | $10^5$ | RPMI-1640 serum free | NR | NR | CSE from two research-grade cigarettes | 6 µL/min; NR | 48 |
| Luo, Y. et al. [33] | Rat Insulinoma cell | INS-1 | NICLR, CAM, China | Intra-CGG | 3D culture | $10^6$ | RPMI-1640 + FBS (15%) + P-S (100 U/mL) + BME matrix | 37 | 5% CO₂ | 1.0 µL/min; NR | 24, 36, 72, 96 |
| Lim, W. and S. Park [34] | Human Carcinoma colorectal Glialblastaoma | HCT116 U87-MG | ATCC | Extra CGG | Spheroid | $2 \times 10^6$ | McCoy’s 5A Medium and Minimum Essential Media + FBS (10%) + P-S (100 U/mL) | 37 | 5% CO₂ | Irinotecan (100 µM) | NR; NR | 72 |
| Jin, D. et al. [35] | Human Endothelial cells | HUVEC | ATCC | Intra-CGG | 2D culture | NR | DMEM/F12 medium + FBS (10%) + P-S (100 U/mL) | 37 | 5% CO₂ | PTX, CDDP, and 5-FU single and mixture | NR; NR | 24 |
| | Human Tumor cells | ACC-M 7) | Dr. Wang (Guangzhou, China) University of Michigan, USA | Extra CGG | Spheroid | $2.5 \times 10^7$ | DMEM/F12 medium + BME matrix | 37 | 5% CO₂ | 10 mmHg/24 h | NR; NR | 2–8 |
| | Human | UM-SCC-6 cells 5) | | Extra CGG | Spheroid | $2.5 \times 10^7$ | High-glucose DMEM + FBS (10%) + P-S (100 U/mL) + BME matrix | 37 | 5% CO₂ | 10 mmHg/24 h | NR; NR | 2–8 |
| Hong, B. et al. [36] | Human Epithelial cervical carcinoma cells | HeLa | NR | Intra-CGG | 3D culture | $10^6$ | DMEM + FBS (10%) + P-S (100 U/mL) + collagen type I | 37 | 5% CO₂ | DOX (200 µg/mL) | NR; NR | 2–8 |
| Ying, L. et al. [37] | Lung Tumor cell | A549 | | | | | | | | | | | |
| | Human Fibroblast cell | HFL1 | | Cell Bank of Type Culture Collection of CAS, China | Intra-CGG | 3D co-culture | $10^6$ | RPMI 1640 and IMDM + FBS (10%) + P-S (100 U/mL) + BME matrix | 37 | 5% CO₂ | PTX; PTX + CAF; PTX + PI3K inhibitor; PTX + GRP78 inhibitor; PTX + CAF + PI3K inhibitor; PTX + CAF + GRP78 inhibitor; | 10 mmHg/24 h; NR | 24 |
| Study | Biological Model | Culture Environment | Toxicity Conditions |
|-------|-----------------|---------------------|---------------------|
| **Ju, S. M. et al. [38]** | Human Tumor liver cell HepG2 | Korean Cell line Bank, Korea Intra-CGG 2D culture 2 × 10⁶ DMEM + FBS (10%) + P-S (100 U/mL) + fibronectin 37 5% CO₂ APAP 1.7 µL/min; NR 24 | |
| **Pasirayi, G. et al. [39]** | Human Breast tumor MCF-7 | Northern Institute for Cancer Research, Newcastle University Intra-CGG 2D culture 2 × 10⁶ EMEM + Gln (2 mM/L) + nonessential amino acids (1%), FBS (10%) + P-S (100 U/mL) + A (1%) + fibronectin (100 µg/mL) 37 5% CO₂ PCN (100 µM); PTX and aspirin 3.5–5 µL/min with 4 h intervals over a period of 24 h; NR 6 | |
| **Li, E. et al. [40]** | Human Bronchial epithelial carcinoma cell Primary Patients of the First Affiliated Hospital of Dalian Medical University Intra-CGG 2D culture 10⁶ Fresh medium 37 5% CO₂ CSE 5–7 µL/min; NR 48 | |
| **Kwapisiewska, K. et al. [41]** | Human Colon carcinoma cells HT-29 | ATCC Intra-CGG Spheroids 1 × 10⁶–5 × 10⁶ RPMI medium + FBS (5%) + L-Gln (1% of 25 mM) + S-P (1%) EMEM medium + FBS (10%) + L-Gln (1% of 25 mM) + S-P (1%) 37 5% CO₂ 5-FU 4.5 µL/min; change medium for 15 min 24 | |
| **Fernandes, J. T. S. et al. [42]** | Saccharomyces cerevisiae Yeast cells VSY72 | NR Intra-CGG 2D culture 1.5 × 10⁷ SC + RAF (yeast nitrogen base without amino acids, 6.7 g/L); RAF (10 µg/L); CSM without URA-TRP³ 30 NR Ascorbic acid 0.3–0.5 µL/min; NR 5 | |
| **Jastrzębska, E. et al. [43]** | Human Lung carcinoma cell A549 cell Embryo cell J3T3 cells ATCC Intra-CGG 2D culture 1 × 10⁶ NR 37 5% CO₂ Cellex (120 µM) and 5-FU (300 µM) single and mixture 15 µL/min; change media 1.2 µL/min for 50 min 24 or 48 | |
| Study | Biological Model | Culture Environment | Toxicity Conditions |
|-------|------------------|----------------------|---------------------|
| Xu, Y. et al. [44] | Human Epithelial cervical carcinoma cells; Colon carcinoma cells; Epidermoid carcinoma cells; HPV-related endocervical adenocarcinoma | HeLa, RKO, CaSki, SMMC-7721 | 2D culture; Intra-CGG | 1.5 × 10^6 | DMEM + FBS (10%) | 37°C | 5% CO₂ | CDDP (0–20 µM) | 4 µL/min; NR | 24–48 h |
| Yang, C. G. et al. [45] | Human Uterine cervix cancer cell | HeLa, Key Lab of Cell Biology of Ministry of Public Health, PRC | Intra-CGG | 2D culture; 10^6 cells/mL | DMEM + FBS (10%) + S-P (100 U/mL) | 37°C | 5% CO₂ | 5-FU (600 mg/mL) and CP (400 mg/mL) single and mixture, and CDDP | 2.0 µL/min; NR | 24–48 h |
| Jedrych, E. et al. [46] | Human Lung carcinoma cell | A549, ATCC | Intra-CGG | 2D culture; 1 × 10^6 | RPMI 1640 medium + FBS (10%) + Glutamax (2 mM) + S-P (100 U/mL) + A (250 ng/mL) | 37°C | 5% CO₂ | 5-FU | 1.2 µL/min for 50 min; NR | 24–48 h |

### Zebrafish embryos

| Study | Biological Model | Culture Environment | Toxicity Conditions |
|-------|------------------|----------------------|---------------------|
| Li, Y. et al. [47] | Zebrafish Embryos | School of Life Sciences, SYSU, China | Intra-CGG | 2D culture; 10–12 eggs (3 hpf) | Ultrapure water medium + HNO₃ (0.1 mol/L) + NaOH (0.1 mol/L) | 28.5 | O₂ | PbAc (1 mg/L); CuSO₄ (0.1 mg/L) | 10 µL/min–5 µL/min to 30 µL/min at each inlet; NR | 48 h |
### Table 3. Cont.

| Study          | Biological Model   | Culture Environment | Toxicity Conditions |
|----------------|--------------------|---------------------|---------------------|
| Yang, F. et al. [45] | Zebrafish Danio rerio | Embryos School of Life Sciences, SYSU, China | Intra-CGG 2D culture 1 embryo/chamber | Embryo medium E3: NaCl (5 mM) + KCl (0.17 mM) + CaCl₂ (0.01 mM) + MgSO₄ (0.16 mM) per 100 mL distilled water | 26 ± 1 Anoxia and normoxia | 
|                |                    |                     |                     | | | 

**Origin**
- NIH: National Institute of Health; Balb/c: Bagg Albino Mouse; E. coli: Escherichia coli; S. elegans: Caenorhabditis elegans; S. pseudintermedius: Staphylococcus pseudintermedius; S. aureus: Staphylococcus aureus; HPV: human papillomavirus; GFP: Green Fluorescent Protein; RFP: Red Fluorescent Protein; BL21(DE3): Ampicillin-resistant Gram-negative E. coli; MRSP: Methicillin-resistant S. pseudintermedius; cll-2: chlorophyll type; P: Subcordiformis: Platymonas Subcordiformis; P: helgolandica: Platymonas helgolandica; ESC-WT: embryonic stem cell wild type; A315T: ESC mutant; RPTECs: renal proximal tubule epithelial cells; PCECs: peritubular capillary endothelial cells; A431-DPNTP: epidermoid carcinoma wild type; A431-S2849GDP: A431-targeted GFP-E-cadherin cells; NIH 3T3: NIH/Swiss mouse embryonic fibroblast cell line; HBE: human bronchial epithelial cells; INS-1: rat insulinoma cell line; HCT116: colon cancer cell line; U87: glioma cell line; HUVEC: human umbilical vein endothelial cell; ACC-M: adenoid cystic carcinoma cell line; UM-SCC-6: human tongue squamous cell carcinoma cell line; HeLa: immortal cervical cancer cell line; A549: adenocarcinomic human alveolar basal epithelial cell line; HFL: human fetal lung fibroblast; HepG2: hepatocellular carcinoma cell line; MCF-2: Michigan Cancer Foundation 7—human breast metastatic adenocarcinoma cell line; HT-29: human colorectal adenocarcinoma cell line; PC3: human prostate adenocarcinoma cell line; K562: human chronic myelogenous leukemia cell line; Jurkat: human T-cell leukemia cell line; RKO: poorly differentiated colon carcinoma cell line; CaSki: human papillomavirus type 16-positive cell line; SMMC-7721: hepatocellular carcinoma cell line; NSCLC: non-small-cell lung cancer cell line; HCC: hepatocellular carcinoma cell line; MCF-7: Michigan Cancer Foundation 7—human breast metastatic adenocarcinoma cell line; MDA-MB-231: human breast adenocarcinoma cell line; HuH-7: human hepatocellular carcinoma cell line; HepG2: human hepatocellular carcinoma cell line; MDA-MB-231: human breast adenocarcinoma cell line; H1299: lung adenocarcinoma cell line; H226: lung adenocarcinoma cell line; SK-BR-3: human breast adenocarcinoma cell line; HT-29: human colorectal adenocarcinoma cell line; A549: adenocarcinomic human alveolar basal epithelial cell line; U87: glioblastoma multiforme cell line; HCT116: colon cancer cell line; U251: glioblastoma multiforme cell line; MDA-MB-231: human breast adenocarcinoma cell line; A549: human adenocarcinomic human alveolar basal epithelial cell line; PC3: human prostate adenocarcinoma cell line; MCF-7: human breast adenocarcinoma cell line; HeLa: human immortal cervical squamous carcinoma cell line; ACC-M: adenoid cystic carcinoma cell line; UM-SCC-6: human tongue squamous cell carcinoma cell line; HeLa: immortal cervical cancer cell line; A549: adenocarcinomic human alveolar basal epithelial cell line; HFL: human fetal lung fibroblast; HepG2: hepatocellular carcinoma cell line; MCF-2: Michigan Cancer Foundation 7—human breast metastatic adenocarcinoma cell line; HT-29: human colorectal adenocarcinoma cell line; PC3: human prostate adenocarcinoma cell line; K562: human chronic myelogenous leukemia cell line; Jurkat: human T-cell leukemia cell line; RKO: poorly differentiated colon carcinoma cell line; CaSki: human papillomavirus type 16-positive cell line; SMMC-7721: hepatocellular carcinoma cell line; NSCLC: non-small-cell lung cancer cell line; HCC: hepatocellular carcinoma cell line; MCF-7: Michigan Cancer Foundation 7—human breast metastatic adenocarcinoma cell line; MDA-MB-231: human breast adenocarcinoma cell line; HuH-7: human hepatocellular carcinoma cell line; HepG2: human hepatocellular carcinoma cell line; MDA-MB-231: human breast adenocarcinoma cell line; H1299: lung adenocarcinoma cell line; H226: lung adenocarcinoma cell line; SK-BR-3: human breast adenocarcinoma cell line; HT-29: human colorectal adenocarcinoma cell line; A549: human adenocarcinomic human alveolar basal epithelial cell line; PC3: human prostate adenocarcinoma cell line; MCF-7: human breast adenocarcinoma cell line; HeLa: human immortal cervical squamous carcinoma cell line; ACC-M: adenoid cystic carcinoma cell line; UM-SCC-6: human tongue squamous cell carcinoma cell line; HeLa: immortal cervical cancer cell line; A549: adenocarcinomic human alveolar basal epithelial cell line; HFL: human fetal lung fibroblast; HepG2: hepatocellular carcinoma cell line; MCF-2: Michigan Cancer Foundation 7—human breast metastatic adenocarcinoma cell line; HT-29: human colorectal adenocarcinoma cell line; PC3: human prostate adenocarcinoma cell line; K562: human chronic myelogenous leukemia cell line; Jurkat: human T-cell leukemia cell line; RKO: poorly differentiated colon carcinoma cell line; CaSki: human papillomavirus type 16-positive cell line; SMMC-7721: hepatocellular carcinoma cell line; NSCLC: non-small-cell lung cancer cell line; HCC: hepatocellular carcinoma cell line; MCF-7: Michigan Cancer Foundation 7—human breast metastatic adenocarcinoma cell line; MDA-MB-231: human breast adenocarcinoma cell line; HuH-7: human hepatocellular carcinoma cell line; HepG2: human hepatocellular carcinoma cell line; MDA-MB-231: human breast adenocarcinoma cell line; H1299: lung adenocarcinoma cell line; H226: lung adenocarcinoma cell line; SK-BR-3: human breast adenocarcinoma cell line; HT-29: human colorectal adenocarcinoma cell line; A549: human adenocarcinomic human alveolar basal epithelial cell line; PC3: human prostate adenocarcinoma cell line; MCF-7: human breast adenocarcinoma cell line; HeLa: human immortal cervical squamous carcinoma cell line; ACC-M: adenoid cystic carcinoma cell line; UM-SCC-6: human tongue squamous cell carcinoma cell line; HeLa: immortal cervical cancer cell line; A549: adenocarcinomic human alveolar basal epithelial cell line; HFL: human fetal lung fibroblast; HepG2: hepatocellular carcinoma cell line; MCF-2: Michigan Cancer Foundation 7—human breast metastatic adenocarcinoma cell line; HT-29: human colorectal adenocarcinoma cell line; PC3: human prostate adenocarcinoma cell line; K562: human chronic myelogenous leukemia cell line; Jurkat: human T-cell leukemia cell line; RKO: poorly differentiated colon carcinoma cell line; CaSki: human papillomavirus type 16-positive cell line; SMMC-7721: hepatocellular carcinoma cell line; NSCLC: non-small-cell lung cancer cell line; HCC: hepatocellular carcinoma cell line; MCF-7: Michigan Cancer Foundation 7—human breast metastatic adenocarcinoma cell line; MDA-MB-231: human breast adenocarcinoma cell line; HuH-7: human hepatocellular carcinoma cell line; HepG2: human hepatocellular carcinoma cell line; MDA-MB-231: human breast adenocarcinoma cell line; H1299: lung adenocarcinoma cell line; H226: lung adenocarcinoma cell line; SK-BR-3: human breast adenocarcinoma cell line; HT-29: human colorectal adenocarcinoma cell line; A549: human adenocarcinomic human alveolar basal epithelial cell line; PC3: human prostate adenocarcinoma cell line; MCF-7: human breast adenocarcinoma cell line; HeLa: human immortal cervical squamous carcinoma cell line; ACC-M: adenoid cystic carcinoma cell line; UM-SCC-6: human tongue squamous cell carcinoma cell line; HeLa: immortal cervical cancer cell line; A549: adenocarcinomic human alveolar basal epithelial cell line; HFL: human fetal lung fibroblast; HepG2: hepatocellular carcinoma cell line; MCF-2: Michigan Cancer Foundation 7—human breast metastatic adenocarcinoma cell line; HT-29: human colorectal adenocarcinoma cell line; PC3: human prostate adenocarcinoma cell line; K562: human chronic myelogenous leukemia cell line; Jurkat: human T-cell leukemia cell line; RKO: poorly differentiated colon carcinoma cell line; CaSki: human papillomavirus type 16-positive cell line; SMMC-7721: hepatocellular carcinoma cell line; NSCLC: non-small-cell lung cancer cell line; HCC: hepatocellular carcinoma cell line; MCF-7: Michigan Cancer Foundation 7—human breast metastatic adenocarcinoma cell line; MDA-MB-231: human breast adenocarcinoma cell line; HuH-7: human hepatocellular carcinoma cell line; HepG2: human hepatocellular carcinoma cell line; MDA-MB-231: human breast adenocarcinoma cell line; H1299: lung adenocarcinoma cell line; H226: lung adenocarcinoma cell line; SK-BR-3: human breast adenocarcinoma cell line; HT-29: human colorectal adenocarcinoma cell line; A549: human adenocarcinomic human alveolar basal epithelial cell line; PC3: human prostate adenocarcinoma cell line; MCF-7: human breast adenocarcinoma cell line; HeLa: human immortal cervical squamous carcinoma cell line; ACC-M: adenoid cystic carcinoma cell line; UM-SCC-6: human tongue squamous cell carcinoma cell line; HeLa: immortal cervical cancer cell line; A549: adenocarcinomic human alveolar basal epithelial cell line; HFL: human fetal lung fibroblast; HepG2: hepatocellular carcinoma cell line; MCF-2: Michigan Cancer Foundation 7—human breast metastatic adenocarcinoma cell line; HT-29: human colorectal adenocarcinoma cell line; PC3: human prostate adenocar...
Among the marine microalgae studied, 80% were Chlorophyta (green microalgae) [23–27], the most frequently seen species being P. subcordiformis (33.3%) [24–27], P. Helgolandica var. Tsingtaoensis (25%) [25–27], and Chlorella sp. (16.7%) [23,26], all of which are from the previously cited phylum. This model was utilized for evaluating the toxicity of water pollutants, mainly metals and composts, most frequently copper (80%) [24–27], followed by mercury [24,25], cadmium [24,27], lead [25], and zinc [24], as well as other substances, such as sodium hypochlorite [23] and phenol [27]. The flow rates of the pollutant solutions and the exposure times in the toxicity evaluations varied greatly, from 0.1 to 50 µL/min and 1 to 72 h, respectively. These toxicity assays were mainly conducted intra-CGG (80%) [23–25,27], in an F/2 medium (80%) [24–27] (an enriched seawater medium was used in one study [23]), in a 2D arrangement, with an average amount of microalgae of 10^5 individuals or a range between 240 and 580 cells/µL, maintained mainly at 25 °C and in controlled light illumination of 60 µmol photon/m^2/s.

Most of the selected studies used human cells (87.1%) [29,30,32,34–39,41,43–46] as the biological model for chemotherapy toxicity screening, consisting, basically, of different types of carcinoma (77.8%) [30,34–39,41,43–46], with the exception of kidney [29], endothelial [35], bronchial epithelial [32], and fibroblast cells [37], which were not necessarily used for the testing of anticancer drugs. Five studies opted for the use of cells from other organisms, such as embryonic stem cells from mice (9.7%) [28,31,43], insulinoma cells from rats (3.2%) [33], and saccharomyces yeast cells (3.2%) [42]. The employed test substances were, mostly, anticancer drugs, comprising 5-FU (26.3%) [35,41,43,45,46], CDDP (21.1%) [29,35,44,45], PTX (15.8%) [35,37,39], DOX (10.5%) [30,36], and, in lower frequency, Rapamycin [28], Gentamycin (GM) [29], Cyclosporin (CsA) [29], Cimetidine (Cim) [29], Iritocetan [34], Acetaminophen (APAP) [38], Pyocyanin (PCN) [39], and Cyclophosphamide (CP) [45], used at a percentage of 5.3% each, with the exception of cigarette smoke extract (10.5%) [32,39], hydrogen peroxide [31], glucose associated with glipizide [33], ascorbic acid [42], the combination of galactose, raffinose, and iron (III) chloride [42], and Celecoxib [43] (5.3% each), and their flow rates (average of 3.4 µL/min) and time of exposure (from 2 to 168 h) were extremely varied. Only 10.5% of the selected studies cultured the cells’ extra CGG system (Figure 2C) [34,35], that is, in a different layer from the one used for the generation of gradient concentrations or outside microfluidic devices, and the majority applied 2D culture (52.6%) (Figure 2A,I) [31,32,38,39,42–46], followed by 3D coculture (15.8%) (Figure 2G) [29,30,37], spheroids (10.5%) (Figure 2C,D) [34,41], 3D culture (10.5%) [33,36], both 2D culture and spheroids (5.3%) [35], and both 2D and 3D cultures (5.3%) [28], using from 104 to 2.5 × 10^7 cells/mL dispersed mainly in Dulbecco’s modified Eagle medium (DMEM) and its variations (42.1%) [29–31,35,36,38,44,45], followed by Roswell Park Memorial Institute (RPMI-1640) medium [32,33,37,41,44,46], and Eagle’s minimum essential medium (EMEM) [39,41]. All cells were incubated at 37 °C (aside from Saccharomyces [42], which were cultured at 30 °C), with a 5% CO_2 humidified atmosphere.

Only two studies used the zebrafish embryos as the biological model [47,48], culturing them in 2D arrangement and intra-CGG, but with different purposes. One of the studies [47], which was performed with 10 to 12 eggs per chamber, focused on lead acetate and copper sulfate toxicity screening by exposing the embryos to these pollutants for 48 h, using flow rates from 5 to 30 µL/min and incubating them in an aerated ultrapure water medium supplemented with nitric acid and sodium hydroxide at 28.5 °C. The second study [48] was performed with one embryo/chamber for chemotherapy toxicity assessment, the drugs employed being ADM, DOX, 5-FU, and CDDP, as well as vitamin C, in different stages of embryo development (4 to 72 h post-fertilization), with the flow rate of 4 µL/min, with incubation in an E3 embryo medium at 26 °C, alternating between anoxia and normoxia.

3.5. Toxicity Screening Evaluation and Outcome of the CGG Microfluidic Device

Table 4 highlights the main points of the proposal, evaluation, and outcome of the selected studies. The main proposal of studies on the microfluidic devices that used microorganisms (bacteria and nematodes) as a model was to perform an AST with single or
combined (due to the antagonism or synergism effect) drugs with different exposure times, using the MIC value as a reference to compare the results with the gold-standard method, searching for the best efficiency while using the lowest amount of drug possible, as assessed by cell viability fluorescent techniques, as well as the influence of the drug’s concentration on genetic alterations and mutations that lead to drug resistance, an extremely relevant issue nowadays due to widespread misusage of antibiotics. The nematodes were used for different purposes; one study [20] evaluated the behavioral response of the worms by fluorescence imaging in the face of manganese toxicity and the protective effect of natural antioxidants while the other [22] evaluated the effectiveness of certain antibiotics on the treatment of bacterial infection on nematodes, either associated or not associated with natural substances, showing these to be of value when treating the infection.

For marine microalgae, the main concern was the toxicity of chemicals linked to environmental contamination, either individually or in combination. These compounds were assessed using viability and motility techniques, which revealed varying sensitivities between different phytoplankton species. One study [23] concluded that *Chlorella* is more resistant than *Pyramimonas* sp. to NaClO and the other microalgae, indicating the greater resistance of *P. subcordiformis* and *P. helgolandica var. tsingtaoensis* to all metals tested, especially CuSO₄, which was shown to be the most toxic.

Most studies that proposed the screening of antitumor substances in cells from humans and animals assessed its efficiency via the use of different fluorescent dyes associated with cell viability evaluation (Calcein AM/Pi, Hoechst 33342, and Annexin-V-FITC), concluding that the drugs have a time/dose-dependent effect in almost all cases in which a drug was tested singly, and, also that the combination of drugs had better efficacy in lower dosages, with similar results seen for assays performed on Petri dish cultures. Only a few studies [28,29] assessed the effects of chemotherapy (or, in two studies [32,40], cigarette smoke extract) on normal cells, through the evaluation of apoptosis or oxidative stress via a reactive oxygen species (ROS) assay, showing that the toxicity and the malignant transformation of cells depend on the time of exposure. The study by Fernandes [42] was the exception in these approaches, evaluating the α-synuclein (aSyn) production and aggregation in Saccharomyces cerevisiae exposed to iron and ascorbic acid, due to the supposed protective effect of these substances.

Each of the zebrafish embryo investigations had a different objective. By using morphometric and behavioral analysis, one study [47] showed the damage effects of metal in different stages of embryo development. The second study [48] focused on the effects of chemotherapeutics normally used in embryo development and the ability of vitamin C to reduce harm.
Table 4. The proposal, evaluation, and outcome of the CGG microfluidic device studies applied in toxicity screening.

| Study | Study Proposal | Techniques for Evaluation | Outcomes | Microfluidics Advantages |
|-------|----------------|---------------------------|----------|--------------------------|
| **Microorganism** | | | | |
| Zeng, W. et al. [16] | To perform an AST on a microfluidic device with lyophilized antibiotics | GFP fluorescence detection; Alamar Blue | The MIC values obtained in the device were consistent with the gold-standard BMD method tested in E. coli k-12: KAN was 7.8 µg/mL; TAC 3.5 µg/mL; and AMP 4.9 µg/mL, and E. coli 5x showed slightly lower levels | Simple, stable, and controllable operation, needing only simple equipment. The device can be stored for later use. Requires only small samples of the tested substance and very little incubation time. Provides high throughput for multiple AST assays at once |
| Nagy, K. et al. [17] | To study the emergence of resistant bacteria in spatial CIPRO gradients, then to perform the genomic sequencing to identify the key mutations that lead to antibiotic resistance | Fluorescence time-lapse microscopy; genomic sequencing and biofilm assay (96 wells) | Most genes affected in 48-h and 72-h were related to the bacterial envelope (rfaG, rfaE, rfaQ, and rfaC). There were similar mutations (in the marR and rfaG genes) and a 2–4× increase in MIC in cells, even without antibiotics and in antibiotic gradient for 48 h that can be explained by the environmental stress, and at 72 h the MIC was 1–30× higher | Microfluidics mimic the complexity of natural microenvironments for bacterial resistance research, facilitating the evolution of resistance and promoting genetic diversity, even before the antibiotics administration |
| Sweet, E. et al. [18] | To identify optimal drug compositions through MIC values of an AST for the treatment of antibiotic-resistant E. coli bacteria and 3D µ-CGG to allow a symmetrical gradient of fluids combined more than 2 drugs solution at time | Resazurin metabolic indicator and spectrophotometry (OD600) | The bacterial growth response and the drug MIC values were the following ~20% for TAC at ~0.26 mg/L, ~5% for CIPRO at ~50 µg/L, and ~30% for AMK at ~11 mg/L. Lower MIC values increased growth. With combined drugs, an antagonism effect between ~0.34 mg/L of TAC and ~28.8 µg/L of CIPRO occurred, and a synergism effect with ~7.68 mg/L of AMK and ~48.8 µg/L of CIPRO was seen, and the value recommended was ~6.08 mg/L of AMK and ~65.3 µg/L of CIPRO in the infection treatment | Provides higher throughput when compared to traditional assays testing multiple antibiotics. The devices are customizable and can be rapidly and cheaply produced for immediate application in medical routine |
Table 4. Cont.

| Study | Study Proposal | Techniques for Evaluation | Outcomes | Microfluidics Advantages |
|-------|----------------|---------------------------|----------|--------------------------|
| Tang, M. et al. [19] | To generate discrete concentration levels through mixing predefined volumes of sample and diluent at different proportions automatically | Absorbance by spectrophotometry | The number of *E. coli* bacteria did not increase after 3 h of exposure to \( \geq 3.5 \mu g/mL \) of the MIC value of AMP | MIC produces results much more rapidly than traditional methods automatically, saving labor time. When integrated with optical detection units, it is more compact and cheaper than commercial spectrometer-based systems. Able to perform multiple processes simultaneously and has a fully customizable concentration gradient. Semi-automized processes. The microfluidic chamber design permits the formation of restricted habitats for the organisms, the administration of precise chemical stimuli, and their reaction assessment by conventional microscopy due to the optical transparency of the device’s materials. Additionally, it has a low cost, good biocompatibility, and versatility of chip. |
| Zhang, B. et al. [20] | To encapsulate a number of worms into the individual chamber and investigate the diverse behavioral responses to manganese toxicity | Fluorescence images by stereomicroscopy | The worm’s motility impairment was dose- and time-dependent when exposed to manganese; high concentrations can cause effects of DAergic neurodegeneration and cell death in the worms, and the natural antioxidants can protect against manganese-induced toxicity | Semi-automized concentration gradient. Able to perform multiple processes simultaneously and has a fully customizable concentration gradient. Semi-automized processes. The microfluidic chamber design permits the formation of restricted habitats for the organisms, the administration of precise chemical stimuli, and their reaction assessment by conventional microscopy due to the optical transparency of the device’s materials. Additionally, it has a low cost, good biocompatibility, and versatility of chip. |
| DiCicco, M. and Neethirajan S. [21] | To evaluate the in vitro activity of fosfomycin against MRSP biofilms, to determine the MBEC | SYTO 9 dye from a LIVE/DEAD® BacLight™ bacterial viability kit | The MBEC value was 8.6 ± 2.1 \( \mu g/mL \) of FO, and the concentration of FO needed to remediate biofilm-embedded cells of MRSP A12 is 8.1 ± 0.9 \( \mu g/mL \) | Facilitates fast analysis of bacterial resistance, pointing to the correct therapeutic conduct. |
| Study          | Study Proposal                                                                 | Techniques for Evaluation                       | Outcomes                                                                                                                                                                                                                                                                                                                                 | Microfluidics Advantages                                                                                                                                                                                                 |
|---------------|--------------------------------------------------------------------------------|-------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Yang, J. et al. [22] | To perform an in vivo antimicrobial screening assay and investigate antibacterial activity of some compounds of rhubarb (aloe-emodin, rhein, and emodin)  | Plasmalemma fluorescent probe DiI by stereomicroscope. The lifespan is tested by LT<sub>50</sub> | The worm’s LT<sub>50</sub> in M9 buffer; in 20% and 10% of BHI-M9 medium, was 60, 24, and 36 h. Exposure to S. aureus for 36 h exhibited suitable virulence to kill worms. At ≤36 h, some infected worms died due to their intestinal lumen filling with a large number of S. aureus, being killed in 5 days. At 48 h, the optimum AMX treatment time, the infected animals were rescued to varying degrees and treated with different concentrations of AMX (0–100 mg/mL); this was carried out in a dose-dependent manner and increased worm survival by at least 1.5-fold with an MIC of 4.0 mg/mL. Rhubarb inhibited the growth of S. aureus, and their MIC values were 7.5, 16.0, and 6.3 mg/mL, respectively, rescuing infected nematodes 1.0–2.0 fold more often at low concentrations, and killing them in higher concentrations (0.60 mg/mL). | Automized assay. Simultaneous generation of multiple concentrations. Reduces manual labor, reagent consumption, and time of analysis. Simultaneous assessment of antibiotic activity and toxicity of these drugs to the host, in vivo. |
| Wang, Y. et al. [23] | To perform DLD separation associated with the possibility of generating different desired concentrations of NaClO solution, using a single integrated photon counter | Chlorophyll fluorescence                        | Pyramimonas sp. viability decreases rapidly in the first 8 min, 8% after 20 min at 250 ppm of NaClO, and to almost zero at 20 min at 280 ppm. The Chlorella required a 500 ppm NaClO for complete inactivation within 20 min | Rapidly generates accurate concentrations. The device, compared to traditional methods, is more compact, cheaper, and more efficient, allowing the assay to be automized, and does not pollute |
Table 4. Cont.

| Study                        | Study Proposal                                                                 | Techniques for Evaluation       | Outcomes                                                                                                                                                                                                 | Microfluidics Advantages                                                                 |
|------------------------------|--------------------------------------------------------------------------------|---------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------|
| Han, B. et al. [24]          | To assess metals’ toxicity to microalgae (copper, mercury, zinc, and cadmium) alone or in a binary/ternary/quaternary mixture | Brightfield microscope          | P. subcordiformis motility inhibition increased with exposure to the increasing concentration of single pollutants of Cu, Hg, Zn, and Cd for 1 h. Hg was the most toxic, followed by Cu and Cd, and Zn was the least toxic. After 1 h, the metal mixture of Hg, Cu, and Cd with Zn was more damaging than Cu with Zn, Cd, or Hg |
|                              |                                                                                 |                                 | After 2 h, the MOT data of Hg, Pb, Cu, and phenol showed them to be 2, 1.5, 2, and 1.2 times more toxic independently. The Cu and phenol mixture inhibited MOT and VSL in the range from 0 to 2.275 toxic units, being dose-dependent mainly for P. subcordiformis and P. helgolandica var. tsingtaoensis |
|                              |                                                                                 |                                 | P. subcordiformis had the best condition for chemostatic culture (max 15 days). The microalgae growth decrease was dose-dependent on Cu concentration, Chlorella was more sensitive to Cu (EC$_{50}$ of 5.52 µmol/L), and P. helgolandia var. tsingtaoensis was more resistant to Cu (EC$_{50}$ of 20 µmol/L) | Offers higher-throughput alternative to conventional methods and might be employed for other types of assays |
| Zheng, G. et al. [25]        | To assess the marine phytoplankton motility and investigate the pollutants’ toxicity effect (Hg, Pb, Cu, and phenol) | Movement tracking by CASA system: MOT, VCL, VAP, and VSL |                                                                                                                                                                                                                                                                   | Incorporation of multiple technologies in one assay. Offers high throughput, automation, low sample consumption, and shorter times. Automation of image acquisition |
| Zheng, G. et al. [26]        | To assess multibiological model in the Cu toxicity test by measurements of cell division rate and esterase activity | Cell viability by cell autofluorescence and esterase activity by FDA |                                                                                                                                                                                                                                                                   | Simplifies toxicity assays. The device allows for easy customization of culturing conditions. Can also be rapidly fabricated |
## Table 4. Cont.

| Study | Study Proposal | Techniques for Evaluation | Outcomes | Microfluidics Advantages |
|-------|----------------|---------------------------|----------|-------------------------|
| Zheng, G. et al. [27] | To assess the chemostat-based cell immobilization through metals’ (Cu and Cd) toxicity and motility | Bright-field microscope | The microalga motility inhibition was dose-dependent on Cu and Cd; *P. helgolandica var. tsingtaoensis* was more resistant than *P. subcordiformis*, for completed motility inhibition (28.60 versus 8.95 µmol/L of Cu and 357.15 versus 196.45 of Cd) using %MOT, VCL, VAP, and VSL data. Cu had a more toxic effect than Cd | Simplifies and accelerates toxicity assays |

**Tumor cells and other models**

| Study | Study Proposal | Techniques for Evaluation | Outcomes | Microfluidics Advantages |
|-------|----------------|---------------------------|----------|-------------------------|
| Chennampally, P. et al. [28] | To evaluate the effectiveness of rapamycin in rescuing the MN of ALS | Immunostaining for GFP and TDP-43; Western blot | ALS-affected motor neuron survival can be increased by 40.44% in a rapamycin dosage range between 0.4 and 1.0 µM | As it is compatible with traditional techniques, they can be combined to obtain the advantages of both. Enhances throughput and results in the entire assay on only one cell culture. Enables multiple simultaneous tests, and has the capacity to stimulate cells to adopt spatial distribution and morphology similar to those in vivo |
| Yin, L. et al. [29] | To predict the nephrotoxicity induced by CDDP, GM, and CsA in renal chip | Calcein-AM/PI and CCK-8 assay | Cell viability was higher in static than fluidic co-culture condition. The cell viability was dose-dependent for all drugs. Cim neutralized and reduced the toxicity of CDDP, thus improving the survival rate of renal cells | Automation of multiple processes. Studies can be performed on models which reproduce key features of an organ’s physiology. Microfluidic devices can bring standardization, automation, and a reduction in costs to drug assays. They can also accelerate the whole process and lessen the impact of human bias |
| Study             | Study Proposal                                                                 | Techniques for Evaluation                              | Outcomes                                                                                           | Microfluidics Advantages                                                                 |
|------------------|--------------------------------------------------------------------------------|--------------------------------------------------------|---------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------|
| Jaberi, A. et al. [30] | To assess the mechanical and chemical stresses in skin cancer cell DOX        | Live/dead (Calcein AM/ethidium homodimer I)             | Cells showed a well-distributed morphology in the chambers and high viability (95%) without fluid flow. The effect of shear stress slightly reduced cell viability (88%) and also led to an increase in DOX concentration. More stable and precise biochemical gradient by static pressure. Pretreatment of low-dose H2O2 protected NIH 3T3 cells against cytotoxicity. An H2O2 lethal dose results in 27.72% of apoptosis. Pretreatment for 24 h with lethal hydrogen peroxide exposure arrested the apoptosis in a dose-dependent manner. Apoptosis ratio decreased to ~27, ~22, and ~14% with 25, 75, and 175 µM, respectively. 16HBE CSE-induced cell apoptosis was dose-dependent, high doses (%19.86%) promoted cell apoptosis, low doses (%12.28%) promoted less apoptosis and continued cell growth (%80% cell viability). The best concentration for CSE stimulation was 12.25%, and after 15 weeks, some cells displayed condensed nuclei and abnormal nuclear-to-cytoplasmic ratios, atypical mitoses, and later a loss of contact inhibition. These alterations were not apparent in the cells treated with cyclosporine. | Microfluidic devices may offer better conditions for 3D cell culturing and co-culturing. A single, versatile, device suitable for the evaluation of different conditions, while guaranteeing high throughput. Simple operation, without the need for external equipment and easy fabrication. A portable device which provides stable concentration gradients and is suitable for long-term cell culture, due to its low shearing effect. Provided greater efficiency, accuracy, lower time, high throughput, and constant control of microenvironmental conditions via computer programs (automation), simple operation, and low costs of construction compared to traditional methods. Emulates the in vivo cell microenvironment and permits the dynamic observation of their growth. |
| Zhao, X. et al. [31] | To generate a shear-free microenvironment for long-term cell culture and adaptive cytoprotection analysis with a pumpless hydrogen peroxide gradient generator | Apoptosis by Annexin-V-FITC and PI                     |                                                                                                   |                                                                                           |
| Qin, Y. X. et al. [32] | To detect the role of the HHS in CSE-induced malignant transformation of 16HBE | Apoptosis by fluorescence (Hoechst 33342), Western blot |                                                                                                   |                                                                                           |
| Study | Study Proposal | Techniques for Evaluation | Outcomes | Microfluidics Advantages |
|-------|----------------|----------------------------|----------|--------------------------|
| Luo, Y. et al. [33] | To drug screen for diabetes with glipizide in 3D INS-1 high-glucose cell model through the circular CGG | MTT, Calcein-AM/PI, Ultrasensitive Rat Insulin ELISA Assay kit | After 24 h addition of glipizide, the decrease rate of inhibition rate with glipizide concentration was 0.5916 and 0.3183 for 3D and 2D models, respectively, and after 48 h, it was 0.9133 and 0.4817 for 3D and 2D models, respectively. The 3D model was more sensitive than the 2D model and produced a greater insulin production response in diabetes drug screening | High throughput. The use of the 3D cell model, facilitated by the device, was shown to produce better results than the traditional method. Multiple parallel assays can be conducted |
| Lim, W. and S. Park [34] | To develop a µFSCD with a CGG that enables cells to form spheroids and grow in the presence of cancer drug gradients | Live/dead | The HCT116 cells' viabilities are drug dose dependent, their viability decrease (63%) after 5 days of 5 μM irinotecan treatment (highest concentration), while the cell viability in the control was 98%. | The device facilitates homogenous spheroid generation. Allows for high-throughput and multiple parallel assays. Its CGG system makes the generated concentrations easily calculable. The materials used allow the gradients formed to be maintained for long periods and the observation to be made using a conventional optical microscope |
| Study                  | Study Proposal                                                                 | Techniques for Evaluation          | Outcomes                                                                 | Microfluidics Advantages                                                                 |
|-----------------------|--------------------------------------------------------------------------------|------------------------------------|--------------------------------------------------------------------------|------------------------------------------------------------------------------------------|
| Jin, D. et al. [35]   | To assess drug sensitivity in spheroid head and neck perivascular tumor model and toxicity in endothelium | Hoechst 33342, PI, and immunostaining | The IC<sub>50</sub> values of PTX, CDDP, and 5-FU for 3D-UM-SCC6 were 0.54, 5.5, and 454 µg/mL, respectively, and for ACC-M, they were 0.45, 5.2, and 400 µg/mL, respectively, being higher than in 2D culture. Low concentrations of PTX or 5-FU combined with CDDP had similar effects to high concentrations of a single drug on tumor cells and low cytotoxicity to HUVEC, leading to ~50% apoptosis of tumor cells, and already high concentrations of combinations were toxic to HUVEC cells. Different patients' tumor cells showed relatively high sensitivities to both combinations with ~60% survival, while others showed low sensitivity with 80% cell survival. | Allows for in vivo administration of drugs to be emulated. Microfluidic devices are better suited for the culture of spheroids, providing better results than conventional 2D culture methods. High throughput, lower costs, maintenance of concentration gradients for long periods of time, and real time analysis are features provided by the microdevice. If needed, more than one drug could be loaded into the device for testing. |
| Hong, B. et al. [36]  | To drug screen with CGG on a paper-based device                                  | Live/dead (Calcein AM/PI; Prestoblue) | After 8 h, the cell viability was >50% with 50 µg/mL DOX and 20% with 200 µg/mL DOX | Allows for multiple simultaneous assays under different drug concentrations to be conducted, as well as automation and a reduction in costs and reagent volumes, increasing the overall efficiency. |
| Ying, L. et al. [37]  | To assess the impact of CAF or HGF on the Met/Pi3K/AKT phosphorylation, GRP78 expression and PTX-induced apoptosis in A549 cells cultured in the 3D matrix | Viability (Rhodamine-123); immunofluorescence; Western blot; immunohistochemistry; apoptosis assay (Hoechst33342 e PI); ELISA | HGF in the CAF matrix activated the Met/Pi3K/AKT and up-regulated GRP78 expression, promoting chemoresistance to PTX-mediated apoptosis in A549 cells. PI3K and GRP78 inhibitors elevated PTX action in cell viability: 90%, 95%, and 100% at 1.28, 2.59, and 4 µM PTX, respectively | High throughput, high sensitivity, reduced substance volumes and overall experiment time. Emulates natural cell microenvironments. |
Table 4. Cont.

| Study | Study Proposal | Techniques for Evaluation | Outcomes | Microfluidics Advantages |
|-------|----------------|---------------------------|----------|-------------------------|
| Ju, S. M. et al. [38] | To investigate APAP cytotoxicity through linear/diffusive-mixing-based CGG on HepG2 cells | Live/dead (Calcein AM/ethidium homodimer 1) | The device showed more sensitivity in toxicity tests than in the 96-well culture (IC₅₀ of 17.8 versus 22.8 mM, respectively), being 128% higher and >1800% less time-consuming due to the use of an automated LabVIEW system that refreshes APAP on the target cells every 4 h | Compared to the 96-well culture system, cells showed higher sensitivity to the substance tested, leading to the conclusion that the microdevice produces more accurate results. Time spent, as well as reagent and sample consumption, are reduced. Provides high throughput, integration of several techniques in one assay, and automation |
| Pasirayi, G. et al. [39] | To chemotherapeutically screen for PCN, PTX, and aspirin singly and combined in two types of tumor cells | Calcein AM | Concentrations of PCN and PTX LC₅₀ on MCF-7 were ~60 and 0.63 µM, higher than in traditional culture (~51 and 0.55 µM), respectively. HepG2 showed the same results with high resistance to PCN (100 µM) with 70% of viability. A total of 0.2 µM of PTX reduced cell viability to 83%, while 4 mM aspirin alone reduced cell viability to 84%. PTX plus aspirin had a higher effect on the loss in cell viability than PTX alone | Cells cultured in the microdevice showed more growth after exposure to drugs, compared to those cultured in 96-well culture plates. Has a low cost and provides the possibility of testing drug combinations |
| Li, E. et al. [40] | To investigate the potential mechanisms underlying tumor-like transformation of continual exposure of primarily cultured human bronchial epithelial cells to CSE | Hoechst33342 and propidium iodide (PI); ROS Assay kit; immunofluorescent assay (GRP78, NF-κB, and PI3K) (E-cadherin and Vimentin); Western blot | Lower doses (2.37–12.28%) of CSE stimulated cell proliferation, but not cell apoptosis, and higher doses (19.86–91.88%) induced cell apoptosis. All analyses were one-way and dose-dependent, as well as the results for ROS | Emulates heavy smoking in humans and the lung microenvironment, making the device ideal for experiments of this kind due to its dimensions, its material properties, and the steady flow of the medium, therefore generating more accurate results. Additionally, it allows for parallel assays with diverse conditions, minimizing possible errors |
### Table 4. Cont.

| Study | Study Proposal | Techniques for Evaluation | Outcomes | Microfluidics Advantages |
|-------|----------------|---------------------------|----------|--------------------------|
| Kwapiszewska, K. et al. [41] | To screen for anticancer drug and chemo-resistance phenomena using the SpheroChip and assessing metabolic activity via dynamic changes in two types of tumor cells | Live/dead (Calcein AM/PI); Fluorescent resorufin (metabolic activity) | The growth of HepG2 spheroids was slightly higher than that of HT-29 inside a chip. HT-29 spheroid had normal metabolic activity until 20% 5-FU (0.125), being resistant to higher concentrations of 5-FU (up to 1 mM) compared to Petri dish culture, and exhibited a strong decrease in metabolic activity of 49% compared to the control (at 24 h) | Provides controllable conditions for 3D culture and the monitoring of the effects of the substances tested for long periods of time, which allows for time-dependent analysis, unlike conventional methods. The device’s fabrication and operation are simple, and it reduces costs and time of experiments |
| Fernandes, J. T. S. et al. [42] | To study aSyn production and aggregation in Saccharomyces cerevisiae using an elastomeric microfluidic device exposed to iron and ascorbic acid | Live-cell imaging; tracking the behavior of single cells by fluorescence image and α-synuclein (aSyn) production | The proportion of single cells trapped was higher for more loosely packed traps (43% for x = y = 20 µm). FeCl₃ induced the formation of aSyn inclusions in a concentration-dependent manner, and ascorbic acid reduced the formation of aSyn inclusions in Y4791 yeast cells | The device enables the creation of controllable microenvironments with precise conditions and, also, the use of a minute quantity of solutions for creation of the concentration gradient, as well as the tracing, over time, of individual cells’ responses, unlike traditional methods. Compared to manual mixing of solutions, the CGG is prone to less mistakes, and is faster and less complicated |
| Jastrzebska, E. et al. [43] | To assess drug combinations of He and 5-FU anticancer on normal mouse embryo cells (Balb/c 3T3) and human lung carcinoma cells (A549) | Live/dead—Calcein AM/PI | Celbx and NSAID inhibited the growth of cancer cells and indicate anticancer properties. After cells’ incubation with Celbx, the viability of A549 cells was lower than normal Balb/c 3T3 cells, and Celbx plus 5-FU enhanced antitumor activity | The CGG made it possible to obtain multiple combinations of the tested substances automatically and simultaneously, while also improving the repeatability |
Table 4. Cont.

| Study                  | Study Proposal                                                                 | Techniques for Evaluation               | Outcomes                                                                                      | Microfluidics Advantages                                                                 |
|------------------------|------------------------------------------------------------------------------|------------------------------------------|------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------|
| Xu, Y. et al. [44]     | To assess on four tumor cell lines (HeLa, CaSki, RKO, and SMMC-7721) and the cytotoxicity of the anticancer drug CDDP | Impedance sensing, fluorescent dye (FICT/PI) | EC_{50} of CDDP for CaSki and SMMC-7721 cells was below 4 \mu M and above 16 \mu M for HeLa and RKO cells. So, CaSki and SMMC-7721 cells showed more severe toxic responses to CDDP treatment compared to the other two cell lines. Cellular morphological changes with the increase in drug concentration: cell shrinkage, increase in cell granularity and chromatin condensation, and the most apoptosis characteristics. The apoptosis effect induced by CDDP was more obvious with the increase in stimulation time and concentration. After 24 h, cell death by 5-FU increased in a concentration/time-dependent manner, inhibiting the survival of both cell types; HT-29 cells were less sensitive than A549 cells. The strongest inhibition, approaching 80% after 48 h of incubation, was observed for A549 cells exposed to 300 \mu M 5-FU. | The device reduces reagent and sample consumption, cost and time of experiment, and enables automation, while providing high-throughput, label-free, and dynamic detection of the effects of substances tested. |
| Yang, C. G. et al. [45]| To assess HeLa apoptosis of the single and combined effects of two drugs through combinatorial, quantitative, and predictable concentration gradient by repeated splitting and mixing | DAPI, annexin V-FITC/PI apoptosis detection kit | The CGG produces an extremely wide range of stable, customizable, and repeatable concentrations. It also possesses a compact design and provides high throughput, while reducing time of analysis. | The device allows for the execution of different methods of toxicological evaluation, as well as automation of processes. It also permits the simultaneous cultivation of cells with different characteristics and the lowering of costs and time needed. |
| Jedrych, E. et al. [46]| To assess the 5-FU cytotoxicity on two human cancer cell lines                  | Calcein AM/PI                              |                                                                                               |                                                                                           |
| Study | Study Proposal | Techniques for Evaluation | Outcomes | Microfluidics Advantages |
|-------|----------------|---------------------------|----------|-------------------------|
| **Zebrafish embryos** | | | | |
| Li, Y. et al. [47] | To perform metal safety evaluations and poison screening using embryos as vertebrate models | Morphological and behavior analyses; body length measured | Pb and Cu revealed an effect at 22 hpf, mortality at 24 hpf, heart rate and body length at 96 hpf, being concentration-dependent. The teratogenicity of Pb and Cu in zebrafish embryos and mixed metals induced more severe toxicity with several types of malformations | NR |
| Yang, F. et al. [48] | To describe a phenotype-based whole-organism model to assess the developmental toxicity and teratogenicity of anticancer drug-induced zebrafish embryos | Stereomicroscopy | ADM and CDDP had similar toxicity and teratogenicity in 4 hpf embryos, and 5-FU was halved under the same conditions. These effects vary according to developmental embryo stages, mainly for DOX, which exhibited obvious time/dose-dependent toxicity and LD 50 = 91.7 µg/mL. The embryos treated with vitamin C were not damaged | Allows for high throughput, combination of technologies, and automation |
As for the advantages of utilizing microfluidic devices rather than traditional macroscale methods, the great majority of the studies reported similar benefits. Firstly, some of the studies reported that the results obtained with microdevices correlate very well with those obtained using conventional methods, sometimes even mimicking more accurately in vivo conditions, showing that the technology in question can be adequately applied when studying toxicity. With that in mind, one of the most important aspects reported is the possibility provided by microfluidic CGG’s ability to generate a very sizeable number of different concentrations (up to 65 in the studies analyzed) in a single device and, consequently, the possibility of conducting multiple parallel assays, both of which, allied with the prospect of automation of processes (such as the generation of the concentration gradient and metabolite collection), can significantly diminish the time expended and make this a high throughput method for toxicity screening. Other very significant advantages brought about by this technology include the small size of the devices, which translates to less space occupied, making it possible to have multiple devices running multiple assays at the same time, further increasing the throughput, and also, the low quantity of reagents used, decreasing the cost of the tests. Besides that, the microdevices can be easily and rapidly fabricated, with different well-established fabrication processes and a variety of materials, and easily operated, as well as integrated with other traditional techniques, combining the advantages of both. Microdevices are also more preferable for 3D cultures than some of the more traditional methods, and they make it possible to create microenvironments that are more like those seen in nature, producing results that are more trustworthy. The analysis of microfluidic devices can also be carried out via a variety of methods, providing the researcher with a lot of design flexibility. The device’s versatility, which allows it to be developed in an endless number of ways for various purposes, with various test chemicals and biological models in mind, is still another significant advantage that can be seen. All these parameters are described in Table 4.

According to their toxicity methodologies and the biological models employed for this testing, the studies’ findings are described in Figure 3 in conjunction with the major features that are considered in this systematic review.
Figure 3. The systematic review identified 4 main types of organisms used for toxicity analysis using the CGG system in microfluidic devices: microalgae, zebrafish embryo, tumor cells and other models, and microorganisms. The figure shows the main important aspects (as percentages) regarding the microfluidic device material, manufacturing technology, gradient system, culture environment, culture site, biological model, and toxicity condition. Abbreviations: PDMS: polydimethylsiloxane; SC: serpentine channel; CGG: concentration gradient generator; RM: red microalgae; CDDP: Cisplatin; 5-FU: 5-Fluorouracil; DOX: Doxorubicin; SM: silicon; SPD-CGG: static-pressure-driven CGG; NR: not reported; Sac-Cer: Saccharomyces cerevisiae; PTX: Paclitaxel; AMP: Ampicillin; TAC: Tetracycline; CIPRO: Ciprofloxacin.

4. Discussion

Advances in microfluidic device development technology for toxicity screening have provided remarkable advantages over conventional two-dimensional cultures due to the reduction in the sample consumption, reaction time, and cost of the operation. This systematic review gave a broad overview of the main aspects and trends regarding the manufacture of microfluidic devices, the promotion of the CGG’s development to boost the effectiveness of its chemical and drug toxicity screening, and the most tried-and-true biological models for addressing issues concerning environment and medical treatments.
Regarding microfluidic device fabrication, all were manufactured in-house, providing device customization for more efficient testing, which was specific to each biological model used. There is still a strong tendency to use materials and manufacturing techniques such as PDMS and soft lithography (91%), but recent articles search for more sophisticated technology, such as 3D printing, silicon micromachining, and direct writing photolithography using glass. PDMS is the most commonly used material in microfluidics, because of its flexibility, biocompatibility, nontoxicity, good stability, and high transparency [49], even though earlier studies—some from more than a decade ago—brought up disadvantages, such as the absorption of small molecules [50], its incompatibility with organic solvents [51], and its vapor permeability [52], and more recent articles have questioned its practicality and widespread use, citing the difficulty of translating results obtained with it to other materials and its poor scalability for commercial purposes [53–56] as major concerns. The studies that did not use PDMS reported the use of materials such as silicon and glass which have, roughly, the same advantages as PDMS beside hydrophilic capabilities, reusability, and flexibility [45]. The biomedical field finds 3D printing to be a highly valuable technology for diagnostic and/or therapeutic purposes; its applications range from tissue engineering to microscale robotics and biosensors, besides rapid prototyping flexibility and a variety of forms and functions, having the advantages of precisely controlling the spatial distribution layer-by-layer, the generation of heterogeneous microorgans, and 3D cellular arrangement on a chip [57,58]. Only one study [39] utilized thermoplastics in some way, which is an interesting finding, given that, in recent years, materials such as polycarbonate (PC), poly(methyl methacrylate) (PMMA), and cyclic olefin copolymer (COC) have been gaining notoriety and have been widely used in industry when aiming for the fabrication of a product [53,54].

The studies that used conventional manufacturing varied the type and number of molds. Photolithography was the most used method of fabrication (79%), mostly due to its high accuracy, despite its high cost [59]. A study on optimization of SU-8 microstructure in high-transparency masks, printed in a photomask, however, showed the possibility of their fabrication with a low-cost process and without the requirement of cleanroom facilities [60]. Laser cutting techniques, as well as CNC, when compared to traditional photolithography and etching methods, have the advantages of being a simple, fast, and direct-write process for the fabrication of different geometrical shapes. Both techniques provide complex geometries with different layers, normally more than one layer (72.3%), with a micrometer scale. The layers represented the different environments and testing functions of the microdevice, providing greater efficiency within its complexity.

Most studies (73%) reported the use of new technologies, aiming to minimize microfluidic problems and also to innovate in the material and manufacturing of microdevices [16–24,27–29,31,32,34–38,41,42,44,47,48]. In addition, some studies proposed technological advances integrating electronic systems (9%) [19,20,31]. The evolution in the fabrication of complex and adaptive microfluidic devices was evidenced in the selected studies with implementations that showed significant advantages of the CGG used, such as its ability to create sophisticated and precisely defined gradient profiles.

The CGG system is a faster and more accurate method for drug and chemical pollutant toxicity analysis. It only needs a small amount of reagent for multiplex analysis, which lowers the cost. It is also capable of screening at the molecular and cellular levels and has multistep liquid-handling capabilities, which is especially useful for complicated screening procedures, in addition to its features of miniaturization, integration, and automation of analytical systems [36,61].

The method of gradient generation was based on two patterns, convective and diffusive. Most of the selected studies used the convective method for gradient generation (67%), which is a simpler and easier method for drawing and calculating. In convection-based gradient generators, the concentration gradient depends on the flow field, which can produce shear stress above the physiological limit endured by cells. The diffusion-based gradient generator, on the other hand, offers isolated chambers due to the interface, and the inside
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reagents are protected from the outside shearing [10]. The Christmas tree generation system was the most used (61%), associated and not associated with other systems, which indicates the frequency of the convective pattern, and its main advantages were its simple design and a well-defined concentration range, allowing isolated assessment of each concentration. However, this pattern can be integrated with other systems such as Y-junction systems or in two separate layers, one in which the convection pattern is evident (the CGG layer), forming the concentrations, and one containing the culture chamber, in which the different concentrations flow through diffusion. A few studies used similar systems to the Christmas tree, such as serpentine channels, cascading mixing, and T-shaped systems, which have certain advantages, for instance, fewer stages [62]. The studies that used the diffusion pattern applied a variety of gradient systems, such as Y-junction, membrane, and droplet generation. This last system shows difficulty in controlling flow and concentration while maintaining the droplet shape, two crucial parameters for toxicity assessment.

Gradient linearity is the expected behavior of CGG, being reported in 91% of studies due to the need to assess dose dependency on drugs and toxicity. The studies used two methods to analyze CGG linearity and performance: flow simulation and validation. The flow simulation occurs in a stage before the CGG manufacturing, allowing quick design adjustment, but only seven studies (21%) reported analysis using the COMSOL software, likely due to its high cost and requirement of an expert user, making access to it difficult. CGG validation is a different type of analysis that can only be performed once the microdevice is complete. The most employed substances for this method were fluorescent agents (55%) [18,20–22,25–28,31,34,35,37,38,42,43,45,47] followed by dye solutions (12%), bringing a visual analysis of the flows of the channels and the concentrations generated [19,29,36,38,39]. Some studies also performed a quantitative analysis to be compared with the final concentrations.

Considering the publication year of the 33 studies included in this systematic review and the different approaches for toxicity screening, the studies from the first five years directed greater attention to environmental problems, such as contamination of the seas by metals and other pollutants (60%) [25–27,35] and advances in treatment with chemotherapeutics, seeking better drug combinations for better efficiency (63%) [35–46], both contexts being analyzed in studies using zebrafish embryos [47,48]. Currently, studies are more focused on effective antitumor therapies (37%) [28–34] and pollutants’ toxicity in the marine microenvironment (40%) [23,24], while the concern over antimicrobial treatments has grown (43% [20–22] to 57% [16–19]).

Among the most tested antibiotics in the toxicity analysis, there was a slight predominance of Ampicillin and Ciprofloxacin, which are effective against a wide range of both Gram-positive and Gram-negative bacteria, while having distinct modes of action [63]. The concentration range of both drugs showed a similar pattern (from 2 to 16), showing MIC values consistent with the gold standard of conventional analysis, being more efficient in terms of analysis time and material consumption, and allowing combined-drug analysis for synergism and antagonism effects, using a drug exposure time from 4 to 72 h. The use of prolonged subtherapeutic levels is a concern regarding bacterial resistance, with microfluidic devices having been shown to be more efficient for this analysis than conventional techniques due to the possibility of mimicking the in vivo microenvironment, while guaranteeing high performance [64]. The main bacteria utilized as a model was the Gram-negative Escherichia coli (E. Coli) (for example, E. Coli k-12), which may cause severe food poisoning and is a global health problem due to the rise in antibiotic resistance. Due to its unrivaled fast growth kinetics, high-cell-density cultures, and quick and simple exogenous DNA transformation, this species of bacteria is the most popular for use in toxicity assays [65]. Almost all bacteria were cultured inside the CGG system in a 2D culture dispersed in a medium from 30 to 37 °C, with the exception of the study by Sweet [18], which cultured them in a separate system (extra CGG), and the studies by Zeng [16], which used 3D culture, and Nagy, who used 3D co-culture [17].
A few studies also used nematode *C. elegans* as a model for toxicity screening, assessing the influence of antibacterial activity with various rhubarb components [22], and dopaminergic neurotoxicity induced or not by manganese associated with antioxidant elements [20]. This is a strong model organism because of its small size, optical transparency, short life cycle, and genetic tractability, among other advantageous traits, such as the ability to be infected by a variety of human pathogens and low cost of maintenance [66]. This microorganism was also cultured inside the CGG system in 2D culture dispersed in the medium from 20 to 25 °C.

Through the evaluation of metals and contaminants, a number of research articles have addressed the problem of environmental toxicity. Global pollutants such as mercury and lead, for instance, have an impact on both human health and the ecology around the world [67]. Microalgae have reportedly been used for biological detoxification, effluent treatment, control of toxic metals in natural waters or effluents, and control of toxic metals in naturally or industrially contaminated waters [68], as well as to retain and immobilize some compounds. Thus, it is essential to create tools that can investigate and aid in the creation of new technologies that are beneficial for the environment and, by extension, for human health and quality of life. Although other metals, such as arsenic (As), cadmium (Cd), chromium (Cr), lead (Pb), and mercury (Hg), are poisonous to microalgae, they can ingest trace amounts of metals, including boron (B), cobalt (Co), copper (Cu), iron (Fe), molybdenum (Mo), manganese (Mn), and zinc (Zn). Low-hazardous metal and compost concentrations can promote the growth and metabolism of microalgae because of the hormesis phenomena [69].

The metals Cu [24–27], Cd [24,27], and Hg [20,24] were evaluated the most often, singly or combined with other chemical elements, likely due to the high plastic accumulation in oceans from unrecycled waste and its decomposition [70] or the increase in mining and industrial activity, leading to mercury deposit [70]. The concentration range of these metals was similar (from 0 to 4 µM), varying from five to eight different concentrations tested. Green microalgae (*Chlorophyta*), the main model used, are photosynthetic protists and one of the groups of algae most closely related to terrestrial plants, also being used as indicators of water quality and having significant ecological importance, as they are components of phytoplankton, one of the primary producers in the food chain [71]. The microalgae were cultured mainly inside the CGG system—with the exception of the study by Zheng [26], which cultured them outside the CGG system—in 2D culture dispersed in the F/2 medium at about 25 °C, in controlled light illumination of 60 µmol photon/m²/s, close to normal environmental conditions.

Zebrafish embryos are frequently used in metal toxicity studies due to their ability to grow outward and having clear enough bodies to be examined under a standard optical microscope [72]. One study in this review evaluated the Pb and Cu toxicity, singly and combined, regarding its teratogenicity in different stages of embryo development, such as the larval, juvenile, and adult stages [47]. Another study used this model to analyze different types of chemotherapy drugs and the protective effect of vitamin C during treatment, evaluating their influence according to the developmental embryo stages [48], using drug doses significantly lower than those applied in the tumor cell and other models group. These embryos were cultured inside the CGG system in 2D culture dispersed in different medium conditions at about 27 °C, and the toxicity evaluation occurred from 1 to 72 h of exposure.

The vast majority of the tumor cell and other model group studies performed antitumor toxicity screening using various tumor cell types from human [26,30–37,39–42] or animals [33] and normal human cells [29,32,35], evaluating, primarily, the effect of the drugs 5-FU [35,41,43,45,46], CDDP [29,35,44,45], PTX [35,37,39], DOX [30,36], isolated or combined with others. CDDP and 5-FU combined are considered the standard antitumor treatment, and PTX followed by CDDP showed greater antitumor activity [73]. The toxicity of isolated Doxorubicin occur via acting on DNA by slowing or stopping the proliferation of cancer cells by inhibiting an enzyme called topoisomerase 2, their cardiotoxicity is the
main factor restricting its use, and the total cumulative dose is the only factor currently utilized to predict toxicity, with microfluidics providing a new form of assessment [74]. 5-FU and CDDP also have activity on DNA, inhibiting thymidylate synthase, and crosslinking with the urine bases on the DNA to form DNA adducts, preventing repair of the DNA, leading to DNA damage and subsequently inducing apoptosis within cancer cells, respectively. The dose of these drugs was significant compared to other drugs, ranging from 0 to 600 mg/mL for 5-FU and 0 to 400 mg/mL for CDDP. PTX has a different anti-tumor mechanism, promoting the assembly of tubulin into microtubules and preventing the dissociation of microtubules, blocking cell cycle progression, preventing mitosis, and inhibiting the growth of cancer cells, being used, in the studies, in lower doses, from 0 to 3.4 mg/mL [75].

These cells were mainly cultured inside the CGG system in 2D conventional culture dispersed in DMEM or RPMI media, being mostly supplemented with fetal bovine serum (FBS) and other supplements at 37 °C. A few studies also used 3D culture and co-culture with different tumor or normal cells, and some studies specified the use of spheroids, a variation of conventional 3D culture. 2D cell culture models have been used to assess the toxicity or efficacy of drug candidates due to the ability to anticipate drug responses, but they have been found to be comparatively weak in comparison to 3D cell cultures, which have better functional and phenotypic characteristics, as well as predictability of therapeutic effectiveness [34,35]. In vivo, cells are arranged spatially into three-dimensional (3D) patterns that are encircled by an extracellular matrix (ECM), which leads to cancer cells growing in 3D cultures; in comparison to 2D cultures, these cells are more resistant to cytotoxic drugs [76]. Spheroids are one of the most relevant and modern models for cancer research. Their morphology and physiology are similar to those of a tumor in vivo, showing a network of cell–cell interactions, a 3D structure, the presence of a natural extracellular matrix, and nutrients, metabolites, and oxygen gradients [77,78].

Other contexts also used normal cells without the influence of chemotherapeutics. Two studies evaluated the influence of dose and time of exposure to tobacco extract on the malignant transformation of normal bronchial cells. The tobacco epidemic is one of the biggest public health threats the world has ever faced; there is no safe level of exposure to tobacco, and chronic cigarette-smoke-induced time-dependent epigenetic alterations can sensitize human bronchial epithelial cells for transformation by a single oncogene [79]. The study by Fernandes [42] investigated the basic molecular effects of aSyn in the context of living cells, with human aSyn being expressed in yeast and found to induce dose-dependent cytotoxicity, while iron (III) chloride and ascorbic acid were shown to have a protective effect [42]. The molecular basis of various human diseases has been extensively researched using Saccharomyces cerevisiae as a model organism. It is most well-studied in eukaryotic cells, while also being the easiest organism to grow under controlled circumstances and to manipulate genetically [80]. The study by Luo [33] used the INS-1 cells, which are a widely used and well-established model for the study of diabetes and their property of glucose-stimulated insulin secretion [81].

A relevant aspect in studies with microfluidics devices evaluated in the biological model was the flow rate used to infuse the nutrients and components to be tested for toxicity. This condition is very important in the biological environment. In microfluidic devices, shear stress is created by fluid flow injection due to several important aspects, such as channel dimensions and geometry, cell concentration, cell line type, and the way the flow rate is delivered, among others. Microfluidics provides a good way to mimic flows found in veins and small arteries, where the flow is usually unidirectional and laminar [82]. Shear stress can influence cell attachment [83], pathological response [84], and developmental biology [85].

The studies’ outcomes in toxicity screening using the CGG system in the microfluidics device showed comparable results to the conventional toxicity studies, and the efficiency evaluation techniques applied were mainly based on fluorescence signals, followed by spectrophotometry and brightfield microscopy, molecular methods, and other techniques
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(enzyme-linked immunosorbent assay—ELISA, 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide—MTT, and Western blot), showing the achievement of high efficiency in a faster way and the possibility of automation.

One of the limitations of this review was the lack of a detailed comparison of the complexity of the structures and geometries presented by the microfluidic devices developed in the studies. This analysis could help us better understand the significance of the micro-CGG on a global scale as well as the role that device design plays in the generation of the gradient and in each biological model that was investigated, but it was challenging due to the wide variation in the geometric arrangement and size of the studied biological models. Another limitation was the time frame used, 10 years, it was not sufficient to confirm whether there was a trend of CGG devices in relation to manufacturing characteristics, and previous gradient generation systems.

This systematic review also identified some methodological problems and research gaps, such as the relationship between the material used to make devices and the biological model or substance tested for toxicity, taking into account the benefits and drawbacks of each material, the sparse use of simulation procedures prior to device fabrication, and also the methodological care with regard to the duration of stable concentrations obtained by the CGG, which may compromise the accuracy of toxicity evaluation.

5. Conclusions

This systematic review showed a variety of toxicity assessment applications in the environmental and medical approaches through concentration gradient generation systems in microfluidic devices. Current studies have adopted new technologies and complex structures to customize the device according to the biological model, to achieve the best testing efficiency and to minimize typical microfluidics issues such as bubbles and shearing. The microfluidic gold-standard technique, soft lithography, using the polymer PDMS, was still the most frequently used, and the Christmas tree shape was the most prevalent CGG design, but alternative techniques and designs were employed to produce a larger variety of concentrations and drug combinations more precisely and more outcomes at once. Thus, the CGG microdevice is an alternative to common pipetting techniques for the evaluation of drugs’/substances’ toxicity in various biological organisms, bringing greater precision with a lower cost.

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References

1. Erhrhrig, E.O.; Ihekwereme, C.P.; Illedigwe, E.E. Advances in acute toxicity testing: Strengths, weaknesses and regulatory acceptance. Interdiscip. Toxicol. 2018, 11, 5–12. [CrossRef]

2. Chapman, K.L.; Holzgreve, H.; Black, L.E.; Brown, M.; Chellman, G.; Copeman, C.; Couch, J.; Creton, S.; Gehen, S.; Hoberman, A.; et al. Pharmaceutical toxicology: Designing studies to reduce animal use, while maximizing human translation. Regul. Toxicol. Pharmacol. 2013, 66, 88–103. [CrossRef]

3. Singh, S.; Khanna, V.K.; Pant, A.B. Chapter 1—Development of In Vitro Toxicology: A Historic Story. In In Vitro Toxicology; Dhawan, A., Kwon, S., Eds.; Academic Press: Cambridge, MA, USA, 2018; pp. 1–19.

4. Saxena, S.; Joshi, R. Microfluidic Devices: Applications and Role of Surface Wettability in Its Fabrication; IntechOpen: London, UK, 2020.

5. Gomez, F.A. The future of microfluidic point-of-care diagnostic devices. Bioanalysis 2012, 5, 1–3. [CrossRef]

6. Nguyen, N.T.; Shaegh, S.A.; Kashaninejad, N.; Pinn, D.T. Design, fabrication and characterization of drug delivery systems based on lab-on-a-chip technology. Adv. Drug Deliv. Rev. 2013, 65, 1403–1419. [CrossRef]

7. Oliveira, A.F.; Pessoa, A.C.S.N.; Bastos, R.G.; de la Torre, L.G. Microfluidic tools toward industrial biotechnology. Biotechnol. Prog. 2016, 32, 1372–1389. [CrossRef]

8. Nur, O.; Willander, M. Chapter 3—Conventional nanofabrication methods. In Low Temperature Chemical Nanofabrication; Nur, O., Willander, M., Eds.; William Andrew Publishing: Norwich, NY, USA, 2020; pp. 49–86.

9. Burklund, A.; Tadimety, A.; Nie, Y.; Hao, N.; Zhang, J.X.J. Chapter One—Advances in microfluidics. In Advances in Clinical Chemistry; Makowski, G.S., Ed.; Elsevier: Amsterdam, The Netherlands, 2020; Volume 95, pp. 1–72.

10. Toh, A.G.G.; Wang, Z.P.; Yang, C.; Nguyen, N.-T. Engineering microfluidic concentration gradient generators for biological applications. Microfluid. Nanofluidics 2014, 16, 1–18. [CrossRef]

11. Ortseifen, V.; Viefhues, M.; Wobbe, L.; Grünberger, A. Microfluidics for Biotechnology: Bridging Gaps to Foster Microfluidic Applications. Front. Bioeng. Biotechnol. 2020, 8, 589074. [CrossRef]

12. Yu, F.; Hunziker, W.; Choudhury, D. Engineering Microfluidic Organoid-on-a-Chip Platforms. Micromachines 2019, 10, 165. [CrossRef]

13. Petreus, T.; Cadogan, E.; Hughes, G.; Smith, A.; Pilla Reddy, V.; O'Connor, M.J.; Critchlow, S.; Ashford, M.; O'Connor, L.O. Tumour-on-chip microfluidic platform for assessment of drug pharmacokinetics and treatment response. Commun. Biol. 2021, 4, 1001. [CrossRef]

14. Kim, S.C.; Cestellos-Blanco, S.; Inoue, K.; Zare, R.N. Miniaturized Antimicrobial Susceptibility Test by Combining Concentration Gradient Generation and Rapid Cell Culturing. Antibiotics 2015, 4, 455–466. [CrossRef]

15. Liberati, A.; Altman, D.G.; Tetzlaff, J.; Mulrow, C.; Ioannidis, J.P.; Clarke, M.; Devereaux, P.; Kleijnen, J.; Moher, D. The PRISMA statement for reporting systematic reviews and meta-analyses of studies that evaluate health care interventions: Explanation and elaboration. PLoS Med. 2009, 6, e1000100. [CrossRef] [PubMed]

16. Zeng, W.; Chen, P.; Li, S.; Sha, Q.; Li, P.; Zeng, X.; Feng, X.; Du, W.; Liu, B.F. Hand-powered vacuum-driven microfluidic gradient generator for high-throughput antimicrobial susceptibility testing. Biosens. Bioelectron. 2022, 205, 114100. [CrossRef] [PubMed]

17. Nagy, K.; Dukic, B.; Hodula, O.; Abraham, A.; Csákávaré, E.; DéR, L.; Wetherington, M.T.; Noorlag, J.; Keymer, J.E.; Galajda, P. Emergence of Resistant Escherichia coli Mutants in Microfluidic On-Chip Antibiotic Gradients. Front. Microbiol. 2012, 3, 820738. [CrossRef] [PubMed]

18. Sweet, E.; Yang, B.; Chen, J.; Vickersman, R.; Lin, Y.; Long, A.; Jacobs, E.; Wu, T.; Mercier, C.; Jew, R.; et al. 3D microfluidic gradient generator for combinational antimicrobial susceptibility testing. Microsyst. Nanoeng. 2020, 6, 92. [CrossRef] [PubMed]

19. Wang, Y.; Huang, X.; Chu, Q.; Ning, X.; Wang, Y.; Kong, S.K.; Zhang, X.; Wang, G.; Ho, H.P. A linear concentration gradient generator based on multi-layered centrifugal microfluidics and its application in antimicrobial susceptibility testing. Lab. Chip 2018, 18, 1452–1460. [CrossRef]

20. Zhang, B.; Li, Y.; He, Q.; Qin, J.; Yu, Y.; Li, X.; Zhang, L.; Yao, M.; Liu, J.; Chen, Z. Microfluidic platform integrated with worm-counting setup for assessing manganese toxicity. Biomicrofluidics 2014, 8, 054110. [CrossRef] [PubMed]

21. DiCicco, M.; Neethirajan, S. An in vitro microfluidic gradient generator platform for antimicrobial testing. Biochip J. 2014, 8, 282–288. [CrossRef]

22. Yang, J.; Chen, Z.; Ching, P.; Shi, Q.; Li, X. An integrated microfluidic platform for evaluating in vivo antimicrobial activity of natural compounds using a whole-animal infection model. Lab. A Chip 2013, 13, 3373–3382. [CrossRef]

23. Wang, Y.; Yang, J.; Zhou, C.; Ding, G.; Chen, M.; Zou, J.; Wang, G.; Kang, Y.; Pan, X. A microfluidic prototype system towards microalgae cell separation, treatment and viability characterization. Sensors 2019, 19, 4940. [CrossRef]

24. Han, B.; Zheng, G.; Wei, J.; Yang, Y.; Lu, L.; Zhang, Q.; Wang, Y. Computer-aided design of microfluidic resistive network using circuit partition and CFD-based optimization and application in microalgae assessment for marine ecological toxicity. Bioprocess Biosyst. Eng. 2019, 42, 785–797. [CrossRef]

25. Zheng, G.X.; Li, Y.J.; Qi, L.L.; Liu, X.M.; Wang, H.; Yu, S.P.; Wang, Y.H. Marine phytoplankton motility sensor integrated into a microfluidic chip for high-throughput pollutant toxicity assessment. Mar. Pollut. Bull. 2014, 8, 147–154. [CrossRef] [PubMed]

26. Zheng, G.; Wang, Y.; Wang, Z.; Zhong, W.; Wang, H.; Li, Y. An integrated microfluidic device in marine microalgae culture for toxicity screening application. Mar. Pollut. Bull. 2013, 72, 231–243. [CrossRef] [PubMed]

27. Zheng, G.; Wang, Y.; Qin, J. Microalgal motility measurement microfluidic chip for toxicity assessment of heavy metals. Anal. Bioanal. Chem. 2012, 404, 3061–3069. [CrossRef]
28. Chennampally, P.; Sayed-Zahid, A.; Soundararajan, P.; Sharp, J.; Cox, G.A.; Collins, S.D.; Smith, R.L. A microfluidic approach to rescue ALS motor neuron degeneration using rapamycin. *Sci. Rep.* **2021**, *11*, 18168. [CrossRef]

29. Yin, L.; Du, G.; Zhang, B.; Zhang, H.; Yin, R.; Zhang, W.; Yang, S.-M. Efficient Drug Screening and Nephrotoxicity Assessment on Co-culture Microfluidic Kidney Chip. *Sci. Rep.* **2020**, *10*, 6568. [CrossRef]

30. Jaberi, A.; Monemian Esfahani, A.; Aghabaglou, F.; Park, J.S.; Ndao, S.; Tamayol, A.; Yang, R. Microfluidic Systems with Embedded Cell Culture Chambers for High-Throughput Biological Assays. *ACS Appl. Bio Mater.* **2020**, *3*, 6661–6671. [CrossRef] [PubMed]

31. Zhao, X.; Yan, X.; Li, Y.; Liu, B.F. Static pressure-driven microfluidic gradient generator for long-term cell culture and adaptive cytopotropism. *Microfluid. Nanofluidics* **2019**, *23*, 62. [CrossRef]

32. Qin, Y.X.; Yang, Z.H.; Du, X.H.; Zhao, H.; Liu, Y.B.; Guo, Z.; Wang, Q. Inhibition of the hedgehog signaling pathway depresses the cigarette smoke-induced malignant transformation of 16HBE cells on a microfluidic chip. *Chin. Med. J.* **2018**, *131*, 1191–1198. [CrossRef]

33. Luo, Y.; Zhang, X.; Li, Y.; Deng, J.; Li, X.; Qu, Y.; Lu, Y.; Liu, T.; Gao, Z.; Lin, B. High-glucose 3D INS-1 cell model combined with a microfluidic circular concentration gradient generator for high throughput screening of drugs against type 2 diabetes. *RSC Adv.* **2018**, *8*, 25409–25416. [CrossRef]

34. Lim, W.; Park, S. A Microfluidic Spheroid Culture Device with a Concentration Gradient Generator for High-Throughput Screening of Drug Efficacy. *Molecules* **2018**, *23*, 3335. [CrossRef]

35. Jin, D.; Ma, X.; Luo, Y.; Fang, S.; Xie, Z.; Li, X.; Qi, D.; Zhang, F.; Kong, J.; Li, J.; et al. Application of a microfluidic-based perivascular system for testing drug sensitivity in head and neck cancers and toxicity in endothelium. *RSC Adv.* **2016**, *6*, 29598–29607. [CrossRef]

36. Hong, B.; Xue, P.; Wu, Y.; Bao, J.; Chuah, Y.J.; Kang, Y. A concentration gradient generator on a paper-based microfluidic chip coupled with cell culture microarray for high-throughput drug screening. *Biomol. Microdevices* **2016**, *18*, 21. [CrossRef] [PubMed]

37. Ying, L.; Zhu, Z.; Xu, Z.; He, T.; Li, E.; Guo, Z.; Liu, F.; Jiang, C.; Wang, Q. Cancer associated fibroblast-derived hepatocyte growth factor inhibits the paclitaxel-induced apoptosis of lung cancer A549 cells by up-regulating the PI3K/Akt and GRP78 signaling on a microfluidic platform. *PLoS ONE* **2015**, *10*, e0129593. [CrossRef] [PubMed]

38. Ju, S.M.; Jang, H.J.; Kim, K.B.; Kim, J. High-Throughput Cytotoxicity Testing System of Acetaminophen Using a Microfluidic Device (MFD) in HepG2 Cells. *J. Toxicol. Environ. Health Part A Curr. Issues* **2015**, *78*, 1063–1072. [CrossRef] [PubMed]

39. Pasirayi, G.; Scott, S.M.; Islam, M.; O’Hare, L.; Bateson, S.; Ali, Z. Low cost microfluidic cell culture array using normally closed valves for cytotoxicity assay. *Talanta* **2014**, *129*, 491–498. [CrossRef]

40. Li, E.; Xu, Z.; Liu, F.; Wang, H.; Wen, J.; Shao, S.; Zhang, L.; Wang, L.; Liu, C.; Lu, J.; et al. Continual exposure to cigarette smoke extracts induces tumor-like transformation of human nontumor bronchial epithelial cells in a microfluidic chip. *J. Thorac. Oncol.* **2014**, *9*, 1091–1100. [CrossRef]

41. Kwapiszewksa, K.; Michalczuk, A.; Rybka, M.; Kwapiszewski, R.; Brzózka, Z. A microfluidic-based platform for tumour spheroid culture, monitoring and drug screening. *Lab Chip* **2014**, *14*, 2096–2104. [CrossRef]

42. Fernandes, J.T.; Teneiro, S.; Gameiro, A.; Chu, V.; Outeiro, T.F.; Conde, J.P. Modulation of alpha-synuclein toxicity in yeast using a novel microfluidic-based gradient generator. *Lab Chip* **2014**, *14*, 3949–3957. [CrossRef]

43. Jastrzębska, E.; Flis, S.; Rakowska, A.; Chudy, M.; Jastrzębski, Z.; Dybkó, A.; Brzózka, Z. A microfluidic system to study the cytotoxic effect of drugs: The combined effect of celecoxib and 5-fluorouracil on normal and cancer cells. *Microchim. Acta* **2013**, *180*, 895–901. [CrossRef]

44. Xu, Y.; Lv, Y.; Wang, L.; Xing, W.; Cheng, J. A microfluidic device with passive air-bubble valves for real-time measurement of dose-dependent drug cytotoxicity through impedance sensing. * Biosens. Bioelectron.* **2012**, *32*, 300–304. [CrossRef]

45. Yang, C.G.; Wu, Y.F.; Xu, Z.R.; Wang, J.H. A radial microfluidic gradient generator coupled with cell culture microarray for high-throughput drug screening. *Biosens. Bioelectron.* **2012**, *33*, 1091–1100. [CrossRef]

46. Jedrzych, E.; Flis, S.; Sofinska, K.; Jastrzębski, Z.; Chudy, M.; Dybkó, A.; Brzózka, Z. Evaluation of cytotoxic effect of 5-fluouracil on human carcinoma cells in microfluidic system. *Sens. Actuators B Chem.* **2011**, *160*, 1544–1551. [CrossRef]

47. Li, Y.; Yang, X.; Chen, Z.; Zhang, B.; Pan, J.; Li, X.; Yang, F.; Sun, D. Comparative toxicity of lead (Pb2+), copper (Cu2+), and mixtures of lead and copper to zebrafish embryos on a microfluidic chip. *Biomicrofluidics* **2015**, *9*, 024105. [CrossRef] [PubMed]

48. Yang, F.; Chen, Z.; Pan, J.; Li, X.; Feng, J.; Yang, H. An integrated microfluidic array system for evaluating toxicity and teratogenicity of drugs on embryonic zebrafish developmental dynamics. *Biomicrofluidics* **2011**, *5*, 024115. [CrossRef]

49. Lin, L.; Chung, C.K. PDMS Microfabrication and Design for Microfluidics and Sustainable Energy Application: Review. *Micromachines* **2021**, *12*, 1350. [CrossRef] [PubMed]

50. Toepke, M.W.; Beebe, D.J. PDMS absorption of small molecules and consequences in microfluidic applications. *Lab Chip* **2006**, *6*, 1484–1486. [CrossRef] [PubMed]

51. Sackmann, E.K.; Fulton, A.L.; Beebe, D.J. The present and future role of microfluidics in biomedical research. *Nature* **2014**, *507*, 181–189. [CrossRef]

52. Mukhopadhyay, R. When PDMS isn’t the best. *Anal. Chem.* **2007**, *79*, 3248–3253. [CrossRef]

53. Cong, H.; Zhang, N. Perspectives in translating microfluidic devices from laboratory prototyping into scale-up production. *Biomicrofluidics* **2022**, *16*, 021301. [CrossRef]

54. Scott, S.; Ali, Z. Fabrication Methods for Microfluidic Devices: An Overview. *Micromachines* **2021**, *12*, 319. [CrossRef]
83. Zhang, X.; Jones, P.; Haswell, S.J. Attachment and detachment of living cells on modified microchannel surfaces in a microfluidic-based lab-on-a-chip system. *Chem. Eng. J.* **2008**, *135*, S82–S88. [CrossRef]

84. Glen, K.; Luu, N.T.; Ross, E.; Buckley, C.D.; Rainger, G.E.; Egginton, S.; Nash, G.B. Modulation of functional responses of endothelial cells linked to angiogenesis and inflammation by shear stress: Differential effects of the mechanotransducer CD31. *J. Cell. Physiol.* **2012**, *227*, 2710–2721. [CrossRef]

85. Kshitiz, K.; Park, J.; Kim, P.; Helen, W.; Engler, A.J.; Levchenko, A.; Kim, D.H. Control of stem cell fate and function by engineering physical microenvironments. *Integr. Biol. Quant. Biosci. Nano Macro* **2012**, *4*, 1008–1018. [CrossRef]