# Mutagens Applied to Microalgae for Random Mutagenesis

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Microalgal biomass and metabolites can be used as a renewable source of nutrition, pharmaceuticals and energy to maintain or improve the quality of human life. Microalgae's high volumetric productivity and low impact on the environment make them a promising raw material in terms of both ecology and economics. To optimize biotechnological processes with microalgae, improving the productivity and robustness of the cell factories is a major step towards economically viable bioprocesses. The success of a random mutagenesis approach using microalgae is determined by multiple factors involving the treatment of the cells before, during and after the mutagenesis procedure. Using photosynthetic microalgae, the supply of light quality and quantity, as well as the supply of carbon and nitrogen, are the most important factors. Besides the environmental conditions, the type of mutagen, its concentration and exposure time are among the main factors affecting the mutation result.

Keywords: random mutagenesis ; algae ; mutagens ; strain development ; microalgal biotechnology

## 1. Physical Mutagens in Microalgal Biotechnology

### 1.1. Ultraviolet Light

UV radiation is mainly used to generate random mutations in microalgal cells. Depending on the wavelength, UV radiation is classified as UV-A (315–380 nm), UV-B (280–315 nm) and UV-C (200–280 nm). As presented in **Figure 1**, UV exposition induces several types of DNA alterations; however, it has to be taken into account that phototrophic cells might be resistant to certain physical mutagens due to their photon-capturing and quenching properties. For instance, *Zygmena circumcarinatum* and *Chlorella protothecoides* revealed a high resistance to ionizing radiation, while *Nostoc* sp., *Stylidium javanicum* and some extremophiles showed UV protective properties [1][2][3][4][5][6].

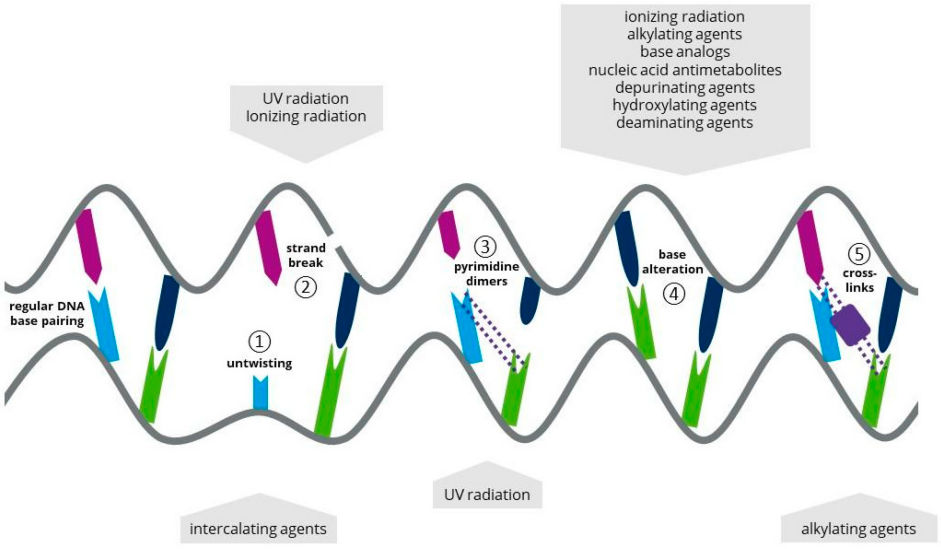


**Figure 1.** Mutagens and their impact on DNA. Five different alterations in DNA are shown: (**1**) DNA strands are untwisted by intercalating agents (chemical mutagen). (**2**) A single- or double-strand break is induced by UV radiation or ionizing radiation (physical mutagens). (**3**) Pyrimidine dimers, covalent binding between two pyrimidine bases, are introduced by UV radiation (physical mutagen). (**4**) Different chemical mutagens can cause base alterations in DNA. (**5**) Cross-links are formed by alkylating agents (chemical mutagen).

Further, 80% of mutation events caused by UV, especially UV-C radiation, are related to the formation of pyrimidine dimers within the DNA. 5-methylcytosine is frequently involved in this type of mutation as it deaminates spontaneously to thymine; hence, the energy absorption shifts to higher wavelengths compared to non-methylated cytosine. Additionally, pyrimidine(6-4)pyrimidone photoproducts can be formed by UV radiation with neighboring pyrimidines between positions 6 and 4 [7][8]. Radiation at 260 nm (UV-C) leads to the most efficient formation of cyclobutene pyrimidine dimers and 6-4-photoreaction products, as DNA absorption reaches its maximum level at this spectral range. Therefore, UV-C irradiation has been recommended for random mutagenesis approaches, including microalgae [9]. A comprehensive overview on UV-radiation-induced mutagenesis approaches is presented in **Table 1**.

### 1.2. Ionizing Radiation

Ionizing Radiation, such as gamma irradiation, X-rays or ion beams, can also act as physical mutagens [10]. Due to the higher energy density compared to UV radiation, ionizing radiation causes serious genetic damages [11], such as the ionization of molecules, the alteration of bases, the breaking of phosphodiester bonds and the production of chromosomal aberrations, such as deletions, translocations and chromosomal fragmentation [12].

In view of the lack of knowledge on interactions between gamma radiation and microalgae, Gomes et al. [13], investigated the effects of various gamma ray intensities on the green alga *Chlamydomonas reinhardtii*, revealing modifications to the PSII energy transfer and a decrease in photosynthetic activity due to the induced formation of reactive oxygen species (ROS) by gamma radiation. Senthamilselvi and Kalaiselvi [14], analyzed the effects of gamma radiation on the microalgae *Chlorella* sp. in a range of 100 Gy to 1100 Gy, showing a 1.4-fold increase in the intracellular neutral lipid content compared to the wild type. Even the biomass production increased in 10 out of 12 mutants compared to the wild type by up to 27.16%.

### 1.3. Atmospheric and Room Temperature Plasma

New physical mutagenesis approaches have been recently presented using atmospheric and room temperature plasma (ARTP) for several bacterial and microalgal strains [15]. ARTP approaches involve the exposition of cells to charged particles [16], electromagnetic fields [17], neutral reactive species [18] and heat [19]. Due to low, controllable gas temperatures, the rapid performance, the high diversity of mutants and the tool's environmentally friendly operation, ARTP mutagenesis shows high potential; however [20], comprehensive datasets, including survival rates of cells or the mutation rate, are not available yet [15].

### 1.4. Laser Radiation

The use of laser radiation in the near infrared and visible spectrum has already been reported for fungi and bacteria [21]. In recent years, it has also been adapted for microalgae. Due to natural heat dissipation and fluorescence quenching, many microalgae show a higher tolerance to radiation in the visible light spectrum. For a significant mutagenesis effect, higher intensity has been realized by using lasers, including semiconductor lasers (632.8 nm), (He-Ne) lasers (808 nm) or Nd:YAG lasers (1064 nm). This mutagenesis approach provides short-term exposure of microalgae in the minute range. Due to the ease of application and the good results obtained in initial studies, e.g., for the improvement in lipid production, there still seems to be potential [22][23]. **Table 1** provides an overview on physical mutagens applied to microalgae.

**Table 1.** Physical mutagens applied to microalgae.

| Mutagen | Method, Exposure Time, Source, Distance, Recovery Time | Reference Microalgae | Mutation Results | | | References |
|---|---|---|---|---|---|---|
| | | | Mutated trait | WT * | M ** | |
| UV | UV 18 W, for 13 min, 15 cm, 24 h darkness | *Chlorella vulgaris* Y-019 | neutral lipid accumulation [g/g dry wt] | 0.11 | 0.26 | [24] |

| Mutagen | Method, Exposure Time, Source, Distance, Recovery Time | Reference Microalgae | Mutation Results | | | References |
|---|---|---|---|---|---|---|
| UV-C | UV-C 253.7 nm, 30-W, 3–30 min, 9 cm, 24 h darkness | *Chlorella* sp. | protein content [g/L] | 0.0242 | 0.0688 | [25] |
| | UV-C 254 nm 1.4 mW/cm$^2$ for 60 s, 15 cm, 16 h darkness | *Chlorella vulgaris* | fatty acids 16:0;18:0, 20:0 [% of total fatty acids] | 27.9; 3.9; 11.9 | 47.4; 5.9; 19.9 | [26] |
| | UV-C 254 nm, 15 W, (Vilber–Lourmat, France), for 30–180 s, 5 cm, 24 h darkness | natural isolates of photosynthetic microorganism | lipid content though Nile red autofluorescence; with fluorescence emission | 35; 1081 | 983; 89,770 | [27] |
| | UV-C 40,000 µJ/cm, 254 nm, overnight darkness | *Scenedesmus obliquus* | trans-fatty acid productivity [g/(L·d)] | 0.095 | 0.112 | [28] |
| | UV-C 254 nm 340 mW cm$^2$, for 3–32 min, 13.5 cm, 24 h darkness | *Isochrysis affinis galbana* | total fatty acid [g/g dry wt] | 0.262 | 0.409 | [29] |
| | UV-C, for 1–10 min, 40 cm, overnight darkness | *Chlorella vulgaris* | lipid content [g/g] | 0.58 | 0.75 | [30] |
| Gamma irradiation | 10 doses of irradiation 50–7000 kGy, 60Co gamma ray irradiator, room temperature | *Scenedesmus* sp. | lipid productivity [g/L·d] | 0.0648 | 0.097 | [31] |
| ARTP | He RF power 100 W, plasma temperature 25–35 °C, for 20; 40; 60 and 80 s, 2 mm | *Spirulina platensis* | Carbohydrates productivity [g/L·d] | 0.0157 | 0.026 | [15] |
| | He RF power 100 W, plasma temperature 25–35 °C, 20–60 s, 2 mm | *Chlamydomonas reinhardtii* | $H_2$ production [mL/L] | ~16.1 | 84.1 | [32] |
| | He RF power 150 W, for 100 s | *Crypthecodinium cohnii* | biomass concentration [g dry wt/L] | 3.60 | 4.24 | [33] |
| Heavy ion beam | $^{12}C^{6+}$ ion beam 31 keVµm$^{-1}$ 160 Gy, | *Nannochloropsis oceanica* | lipid productivity [g/L·d] | 0.211 | 0.295 | [34] |
| | $^{12}C^{6+}$ ion beam, 90 Gy | *Desmodesmus sp.* | lipid productivity [g/L·d] | 0.247 | 0.298 | [35] |
| Low-energy ion beam implementation | N+ ion beam chamber pressure $10^{-2}$ Pa Dose of implantation $0.3–3.3·10^{15}$ ions cm$^{-2}$ s$^{-1}$ | *Chlorella pyrenoidosa* | lipid productivity [g/L·d]; Lipid content [g/g dry wt] | 47.7; 0.337 | 64.4; 0.446 | [36] |

| Mutagen | Method, Exposure Time, Source, Distance, Recovery Time | Reference Microalgae | Mutation Results | | | References |
|---|---|---|---|---|---|---|
| laser radiation | He–Ne laser 808 nm, 6 W, 4 min, 24 h darkness | *C. pyrenoidesa* | lipid content [g/g dry wt] | 0.354 | 0.780 | [22] |
| | Nd:YAG laser 1064 nm, 40 mW 8 min, 24 h darkness | *Chlorella vulgaris* | lipid content [g/g dry wt] | 0.315 | 0.525 | [22] |
| | Nd:YAG laser 1064 nm, 40 mW 2 min, 24 h darkness | *Chlorella pacifica* | lipid content [g $L^{-1}$] | 0.033 | 0.088 | [37] |
| | semiconductor laser 632 nm, 40 mW, 4 min, 24 h darkness | *Chlorella pacifica* | lipid content [g $L^{-1}$] | 0.033 | 0.077 | [37] |

* Wildtype, ** Mutant.

# 2. Chemical Mutagens in Microalgal Biotechnology

## 2.1. Alkylating Agents as a Chemical Mutagen

Alkylating agents (AAs) are commonly used in random mutagenesis to induce nucleotide substitutions within the DNA. AAs transfer alkyl residues, predominantly methyl and ethyl groups, yielding a change in base pairing, followed by typical point mutations after replication of the DNA. It was observed that chloroethylating drugs can also cause sister chromatid exchange or DNA breaks [38], even though AAs cannot induce the direct scission of the DNA backbone [39]. Alkylation leads to the formation of adducts on either O- or N-atoms of nucleotides or O-atoms in phosphodiesters. O-alkylations are particularly potent mutagens, while N-alkylations act predominantly cytotoxic rather than mutagenic [38][40].

One widely used chemical mutagen is ethyl methanesulfonate (EMS), which induces point mutations, in particular, by guanine alkylation, yielding an A·T→G·C transition. Other AAs (shown in **Table 2**) applied to induce random mutations include methylnitronitrosoguanidine (MNNG) [41], diethyl sulfate (DES) [42], N-methyl-N-nitrosourea (NMU) [43] or N-methyl-N′-nitro-nitrosoguanidine (MNNG) [44][45], which can methylate almost all O- and N-atoms, up to several hundred times more effectively than similar concentrations of other monofunctional AAs [39].

AAs have also been used in combination with other mutation approaches, such as exposure to UV radiation (MNNG and EMS) [46][47] or base analogs (MNNG) [39], in order to achieve a higher mutation rate.

## 2.2. Base Analogs (BAs) as a Chemical Mutagen

Chemicals that are capable of replacing DNA bases during the replication process are called base analogs (BA). If the BA is chemically bound to deoxyribose, there is a possibility that it will change shape and, thus, pair with an incorrect base during replication. Depending on the BA used, different types of changes in DNA pairing can be induced [48][49].

5-bromodeoxyuridine (5BrdU) is a uridine/thymidine analog. If 5BrdU is bound to deoxyribose, it is capable of a tautomeric shift to its enol form, leading to a guanine–cytosine-base pairing after DNA replication (A·T → G·C) [50]. Since it changes the structure by tautomeric probability, it can also cause a mutation in the opposite way, pairing with thymine instead of cytosine (G·C → A·T) [51].

2-aminopurine (2AP) is an adenine analog that causes similar changes in DNA pairing to 5BrdU [52]. 5-azacytidine (5AZ) is one of the most commonly used cytidine analogs due to its unique mutagenic specificity, changing only from cytidine BA to a guanine BA (C·G → G·C) [53].

When combined, some BAs have been detected to show a higher mutagenic effect than they could normally accomplish on their own. Combining 2AP and zebularine (ZEB) resulted in a 35-fold increase in mutation frequency in *E. coli* [54]. Similar effects can be observed for the combination of BAs with other physical or chemical mutagens, such as UV radiation and AAs. The repair mechanisms activated by the mutagens increase the probability of the BAs being introduced into the DNA [48]. Similar mechanisms can be assumed using BAs to induce random mutations to microalgae [53]; however, further research is necessary in this field.

### 2.3. Antimetabolites (AMs) as a Chemical Mutagen

The structure of AMs is very similar to metabolites that appear naturally in the cell, but they cannot fulfill their function. AMs, such as 5′fluoro-deoxyuridine (5′FDU) or 2-Desoxy-D-glucose, are inhibiting essential enzymes or mechanisms necessary for DNA replication [55][56]. AMs tend to have multiple mutating and cytotoxic effects, e.g., the pyrimidine analog 5′FDU. After biotransformation, 5′FDU inhibits the enzymatic transformation of cytosine nucleosides into their deoxy derivative and the incorporation of thymidine nucleotides into the DNA strand [56].

AMs have been successfully used as chemical mutagens for many bacteria and fungi species [55][56][57]. In combination with a physical mutagen, such as UV light, good mutagenesis results have been reported in recent studies [55]. However, applying AMs to microalgal cells is a future field of research.

### 2.4. Intercalating Agents (IAs) as a Chemical Mutagen

IAs wedge between the DNA base pairs due to their particular shape. Streisinger et al. [58] recognized that this interaction often occurs in regions with repeated base pairs (e.g., CCCCC) during DNA replication. The bonds are reversible and non-covalent.

This intercalating leads to the deformation of base pairs, resulting in the untwisting and lengthening of the DNA strands. These structural modifications to the DNA affect many functions, such as transcription, replication and repair mechanisms, and may inhibit them or be mutagenic [59].

Acridine and its derivatives are the most widely used and studied DNA IAs. IAs can be mono-intercalators, bis-intercalators or both (such as echinomycin), often depending upon the length of the alkyl chain separating the chromophores [60][61].

Mono-intercalators appear either as frameshift mutations in bacteria or as non-mutagens. Bis-intercalators act as “petite” mutagens, e.g., in *Saccharomyces cerevisiae*, suggesting that they may be more likely to target mitochondrial than nuclear DNA. IA often introduces frameshifting mutations, which they are commonly used for [59]. Petite mutants are described by Ephrussi [62], as cells having defective or altered mitochondrial DNA, resulting in very small (“petite”) colonies [63]. In microalgae and other eukaryotes, IAs seem to introduce mutations, especially in the mitochondrial genome [61][64].

Most IAs, such as echinomycin and acridine and its derivatives, have so far mainly been studied for bacteria, bacteriophage and yeast. A wider use for the random mutagenesis of microalgae is still pending.

### 2.5. Other Approaches for Chemical Mutagenesis

A vast number of other chemicals are described in fundamental biology literature [7][12], for example, deaminating agents (e.g., nitrite) or hydroxylating agents (e.g., hydroxylamine), which replace the amino group of bases with a hydroxyl group and cause alterations in base pairing. Cross-linking agents (e.g., psoralen) or adduct-forming agents (e.g., acetaldehyde) bind covalently to DNA bases and, thus, complicate DNA replication. Other chemical mutagens include mycotoxins (e.g., aflatoxin B1), which can cause indirect damage to metabolites [7][12].

**Table 2.** Chemical mutagens applied on microalgae. * Derived from original data.

| Mutagen | Mutagen Concentration, Time of Exposure | Reference Microalgae | Mutation Results | | | References |
|---|---|---|---|---|---|---|
| | | | Mutated trait | WT * | M ** | |

| Mutagen | Mutagen Concentration, Time of Exposure | Reference Microalgae | Mutation Results | | | References |
|---|---|---|---|---|---|---|
| EMS | EMS 0.1–1.2 M for 60 min | *Nannochloropsis* sp. | fatty acid methyl esters [g/g of dry wt] | 0.123 | 0.238 | [65] |
| | EMS 0.4–1 g/L for 60–120 min | *Haematococcus pluvialis* | total carotenoid; Astaxanthin [g/g of dry wt] | 0.02; 0.005 | 0.02; 0.019 | [66] |
| | EMS 300 mM for 60 min | *Chlorella vulgaris* | protein content [g/g of dry wt] | 0.353 | 0.455 | [67] |
| | EMS 0.2–0.4 M for 2 h in darkness | *Chlorella vulgaris* | violaxanthin [mg/L culture] | 1.64 | 5.23 | [68] |
| | EMS 0.1–0.2 M | *Phaeodactylum tricornutum* | total carotenoids [g/g dry wt] | 0.009 | 0.011 | [69] |
| | EMS 0.2 M for 2 h in the dark | *Dunaliella tertiolecta* | Zeaxanthin [µg/$10^6$·cells] | 0.131 | 0.359 | [70] |
| | EMS 20–40 µL/mL for 2 h | *Chlamydomonas reinhardtii* | fatty acid methyl esters yield [%] | 6.53 | 7.56 | [71] |
| | EMS 0.2 M for 2 h in the dark | *Dunaliella salina* | carotenoid synthesis [Mol Car/Mol Chl] | 0.99 | 1.24 | [72] |
| | EMS 100 µ mol $mL^{-1}$, for 30 min | *Chlorella* sp. | lipid content [g/g of dry wt]; productivity [g/(L·d)] | 0.247; 0.1536 | 0.356; 0.2487 | [73] |
| | EMS 0.4 M, for 60 min | *Coelastrum* sp. | Astaxanthin content [g/L] | 0.0145 | 0.0283 | [74] |
| EMS + UV | UV + EMS 25 mM for 60 min | *Chlorella vulgaris* | lipid content [%] | 100 | 167 | [46] |
| | UV 5–240 s, 245 nm + EMS 0.24 mol/L for 30 min | *Nannochloropsis salina* | fatty acid methyl ester [g/g of dry wt] | 0.175 | 0.787 | [75] |
| MNNG | MNNG 0.1 mM for 60 min | *Haematococcus pluvialis* | Total carotenoid content [g/L] | ~0.067 | 0.089 | [41] |
| | MNNG 5 µg/mL for 60 min | *Chlorella* sp. | max. growth rate under alkaline conditions [ $d^{-1}$] | 0.064 | 0.554 | [76] |
| | MNNG 0.02 mol/L for 60 min | *Nannochloropsis oceanica* | Total lipid content [g/g] Lipid productivity [g/(L·d)] | 0.241; 0.0065 | 0.299; 0.0086 | [50] |
| | MNNG 0.1–0.2 M | *Phaeodactylum tricornutum* | total carotenoids [g/g dry wt] | 0.009 | 0.011 | [69] |
| | MNNG 0.2 mg/mL | *Chlorella sorokiniana* | Lutein content [g/L] | 0.025 | 0.042 | [44] |
| | MNNG 0.25–0.5 mM | *Botryosphaerella* sp. | lipid [g dry wt/($m^2$ day)]; biomass productivity [g dry wt/($m^2$·day)] | 1.0; 3.2 | 1.9; 5.4 | [45] |
| NMU | NMU 5 mM for 60–90 min | *Nannochloropsis oculata* | Total fatty acid [g/g dry wt] | 0.0634 | 0.0762 | [43] |
| DES + UV | UV 7–11 min 254 nm + DES 0.1–1.5% (V/V) 40 min | *Haematococcus pluvialis* | astaxanthin content [mg/L] | ~0.031 | ~0.089 | [42] |
| 5BU | 5BU 1 mM for 48 h | *Chlamydomonas reinhardtii* | $O_2$ tolerance [%] | 100 | 1400 | [77] |
| 5′FDU | 5′FDU 0.25 and 0.50 mM for 1 week | *Chlorella vulgaris* | fatty acids 16:0; 18:0; 20:0 [% of total fatty acids] | 27.9; 3.9; 11.9 | 46.9; 5.5; 18.5 | [26] |
| Acriflavin | Acriflavin 2–8 µg/mL for 1–3 d in darkness | *Chlamydomonas reinhardtii zyklo* | Loss of respiratory rate [nmol $O_2$/(min·$10^7$ cells)] through loss of mitochondrial DNA | 23.2 | 3.7 | [64] |

* Wildtype, ** Mutant.

## 3. Further Approaches in Random Mutagenesis

Recently, combined mutagenesis approaches have generated high interest as results indicated that they have a higher success rate than individual approaches. For instance, Wang et al. [42] applied a two-step random mutagenesis protocol to *Haematococcus pluvialis* cells using first UV irradiation, then EMS and DES mutagenesis, causing astaxanthin production to increase by a factor of 1.7 compared to the wild strain. Beacham et al. [75] used a reverse protocol for *Nannochloropsis salina*, starting with exposure to EMS, followed by UV irradiation, yielding a three-fold increase in cellular lipid accumulation. Comparable results were achieved by Sivaramakrishnan and Incharoensakdi [78], who exposed *Scenedesmus* sp. to UV irradiation in combination with oxidative stress by $H_2O_2$.

Other approaches can be used to select desired microalgal cells if the results obtained by random mutagenesis are insufficient. Among them, Adaptive Laboratory Evolution (ALE) is commonly used to adapt the physiology of cells to specific process conditions, such as high temperatures [79]. Its principle is based on natural selection, as presented in the Darwinian Theory, on the laboratory bench [80], and includes extensive cultivation in a specifically designed lab environment so that enhanced phenotypes can be selected after a long period of time [81]. The environmental conditions that can be altered include light irradiation, lack of nutrients, such as nitrogen, osmotic, temperature and oxidative stress [80][82][83]. Connecting the results of ALE with whole genome sequencing and “omics” methods enables gene functions to be discovered easily [81]. However, ALE does not prevent gene instability that might occur more often than in randomly mutated cells [79][82].

Additional environmental factors can be applied on microalgae; for example, Miazek et al. [84] reviewed the use of metals, metalloids and metallic nanoparticles to enhance cell characteristics. Moreover, phytohormones or chemicals acting as metabolic precursors have already been applied to microalgae [85]. A discussion of the methods used in the latter case exceeds the scope of this research.

More recently, a new technique was developed, known as Space Mutation Breeding (SMB). This technique may have direct or indirect effects on the growth and metabolic activities of microalgae, due to the unusual environment of space, characterized by high-energy ionic radiation, space’s magnetic field, ultra-high vacuum and microgravity [86]. The SMB technique provides some advantages, such as the great improvement in species’ qualities in a short time [87]. This was achieved by Chen Zishuo et al. [86], with a seawater *Arthrospira platensis* mutant, yielding a sugar content 62.26% higher than the wild type.

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