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Impact of chemicals and physical stressors on horizontal gene transfer via natural transformation

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Contaminants in the environment can increase natural transformation rates in bacteria. However, the co-occurrence of a large suite of contaminants may result in final transformation rates that are not based on the geometric addition of individual responses. Here we show that the combination of different chemicals and physical stressors results in natural transformation that do not always follow geometric additive responses. Specifically, some combinations increased transformation rates synergistically, while others decreased rates antagonistically. Unpredictability in the natural transformation outcome was also observed when *Acinetobacter baylyi* ADP1 was exposed to chlorinated reclaimed water. We determined that the stimulatory effect of contaminants on natural transformation can be compensated when extracellular DNA concentrations fall below 0.1 ng l⁻¹ per 2 × 10⁷ cells. Thus, wastewater treatment process that can minimize concentrations of extracellular DNA and cell load would be a key intervention strategy to minimize natural transformation frequency arising from the use of treated wastewater.

Extracellular DNA (eDNA) is the genetic material located outside of cells. This is released into the environment by either active or passive extrusion mechanisms or by cell lysis^{1–3}. eDNA is found ubiquitously in various ecosystems (for example, river, soil and wastewater) at concentrations ranging from 8.6 µg l⁻¹ to 78 µg l⁻¹ in treated wastewater, to even higher concentrations of 0.3 to 200 µg g⁻¹ in soils^{4–7}. eDNA can be integrated into the chromosome of a host cell by means of natural transformation. When eDNA is present in fragment sizes that still retain information needed for gene expression, natural transformation may result in the acquisition of functional gene traits by the bacterium host^{8,9}. Natural transformation is a two-step process. First, eDNA binds

to the DNA transporters present on the surface of a competent cell to be translocated to the cytoplasm as single-stranded DNA. Subsequently, the up-taken eDNA is integrated into the bacterial chromosome by homologous recombination in a RecA-dependent process¹⁰. As the process of natural transformation involves both DNA translocation and integration, stressors affecting translocation or integration can potentially contribute to the stimulation/inhibition of natural transformation^{11–13}.

Multiple studies have since confirmed the role of stressors such as pharmaceutical compounds, artificial sweeteners and chlorine disinfection on natural transformation rates^{14–20}. However, most of these

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studies^{16–18} only assessed the first step of natural transformation (that is, translocation) by using a plasmid as the reporter marker. The use of plasmids also does not mimic situations that might be more prevalent in the natural environment. For example, DNA fragmentation proceeds quickly after cell death, and larger fragments such as plasmids are not expected to persist well in aerobic wastewater effluents²¹. Most antibiotic resistance genes range between 400 bp and 2,000 bp (ref. 22) and natural transformation to *Acinetobacter baylyi* can happen with eDNA as short as 20 bp (ref. 23). Hence understanding the natural transformation frequency using short-length donor DNA and a reporter system that can reflect both steps of the natural transformation process would provide a better proxy. Furthermore, the current understanding of how these stressors contribute to eDNA transformation is only based on individual factorial analysis. In a complex matrix like treated wastewater, stressors co-exist and there remains uncertainty on how stressors, when present together, would interact, and affect natural transformation frequencies.

The effects of multi-factorial combinations of stressors on bacterial growth have been studied in the past, particularly those related to the combined use of antibiotics²⁴. Similarly, a recent study on co-exposure of Chinese hamster ovary cells to disinfection byproducts (DBPs) reported an increase in cytotoxicity endpoints consistent with an additive response²⁵. Here we ask if co-exposure of bacterial cells to physical and chemical stressors would also result in a geometric additive outcome of their individual natural transformation frequencies. If this is the case, one can easily predict the extent of horizontal gene transfer in wastewater and during water reuse events if the stressors are well characterized, and appropriate intervention strategies that aim to reduce the dissemination of stressors that contribute the most to natural transformation should be undertaken. If this is, however, not the case, then horizontal gene transfer outcomes arising from stressors in wastewater would be hard to predict, and developing intervention strategies that work on the basis of precautionary principle may be more appropriate to protect public and environmental health.

In this Article, we analysed the impact of five different stressors on natural transformation: one physical stressor (that is, solar irradiation), three water disinfection byproducts (that is, chloroform (CHCl₃), formaldehyde (FA) and dibromoacetamide (DBAC)) and one pharmaceutical compound (that is, carbamazepine (CMZ)). We first analysed their individual impact on natural transformation, and then test them in co-incubation to determine their combined impact on natural transformation. We further investigated the mechanisms behind the alterations observed in natural transformation rates by assessing the production of reactive oxygen species (ROS) and mutagenicity of each stressor, their impact on bacterial cell membrane permeability and the gene expression profile of the stressor-treated cells. Our findings suggest that certain co-exposure permutations can lead to antagonistic, neutral or synergistic responses on natural transformation rates, which do not always follow the geometric addition of combined factors. Given the unpredictability of natural transformation outcomes due to the co-presence of stressors, we explored possible solutions to minimize the impact of existing contaminants on horizontal gene transfer in treated water by determining the lowest concentration of eDNA to cell ratio that would render natural transformation frequency similar to background levels. Findings from this study address a knowledge gap in terms of how contaminants can induce natural transformation and aid in developing a potential solution to alleviate concerns when reusing treated wastewater.

Natural transformation response in single-stressor assays

Among the five stressors evaluated, solar irradiance (solar), CMZ and DBAC increased natural transformation rates in a concentration-dependent manner (Fig. 1a–c). Compared with untreated controls,

solar irradiance increased transformation rates by 1.4-, 1.6-, 1.8- and 2.2-fold at 69.6, 111.4, 125.4 and 153.2 mJ cm⁻², respectively ($P < 0.001$, Holm–Bonferroni method). Similarly, compared with untreated controls, concentrations of 0.15, 0.23, 0.28 and 0.32 μM CMZ increased natural transformation by 1.8-, 2.1-, 2.3- and 2.3-fold, respectively ($P < 0.001$, Holm–Bonferroni method). DBAC significantly increased transformation by 2.0- and 2.3-fold at 5 μM and 10 μM, respectively ($P < 0.001$, Holm–Bonferroni method, Fig. 1c). Transformation rates at 20 μM were similar to those of the untreated controls. This might be explained by increased cytotoxicity at this DBAC concentration (Supplementary Fig. 1c). In contrast to DBAC, cells exposed to FA and CHCl₃ exhibited decreased transformation rates in a concentration-dependent manner compared with untreated controls (Fig. 1d,e). FA-treated cultures showed a significant decrease in transformation rates at 500, 600 and 800 μM, resulting in a fold change of 0.7, 0.6 and 0.5 versus untreated controls, respectively ($P < 0.001$, Holm–Bonferroni method). Likewise, cells exposed to CHCl₃ exhibited a general trend of concentration-dependent decrease in transformation with concentrations above 1.6 mM, showing statistically significant changes in transformation frequencies compared with the control ($P < 0.001$, Holm–Bonferroni method). The observed decrease by both FA and CHCl₃ did not associate with cytotoxicity (Supplementary Fig. 1d,e). All tested concentrations did not result in any cytotoxicity compared with the control (Supplementary Figs. 2 and 3).

Mutagenicity endpoints for single stressors

We evaluated mutagenicity endpoints by means of a pre-incubation assay using the tester strain *Salmonella enterica* sv. Typhimurium TA100. The tester strain TA100 has been genetically modified to increase cell permeability and repair mechanisms²⁶, and therefore, mutagenic and cytotoxic thresholds observed in TA100 may not always be representative of other bacteria. However, the Ames Tester strain system remains the current standard assay to determine the mutagenicity of compounds against bacteria²⁶, and our earlier study has shown that a mutagenic response observed in TA100 correlated well with an increase in DNA repair mechanisms observed in *A. baylyi* ADP1, hence signifying DNA damage induced in *A. baylyi* ADP1 (ref. 15,27).

Exposure to solar irradiance of 6.96, 13.9, 27.8, 41.8 and 55.7 mJ cm⁻² led to a significant increase of TA100 revertant by 12.7-, 16.5-, 22.5-, 26.0- and 29.1-fold compared with the untreated TA100 controls (Fig. 2a). Similarly, TA100 exhibited a concentration-dependent increase in the number of revertants observed when exposed to DBAC and FA (Fig. 2c,d). The molecular mode of action of FA is complex, and mutagenic outputs include base-change and frameshift mutations²⁸. It is, therefore, noted that not all mutagens inducing base changes and mutagens would result in a corresponding increase in natural transformation frequency. On the other hand, CMZ and CHCl₃ did not have an effect on the mean of revertant colonies recovered in TA100 cell cultures pre-incubated with these chemical stressors (Fig. 2b,e), suggesting that there may be other mechanisms beyond mutagenicity (for example, cell wall permeability) that is inducing natural transformation frequency for CMZ.

Cell permeability impact due to single stressors

An increase in cell wall permeability can facilitate the translocation of eDNA into the cytoplasm. Hence, we examine if the three stressors, namely solar, CMZ and DBAC, increased natural transformation frequency by means of increasing cell wall permeability. For those stressors that increased natural transformation frequencies (that is, solar, CMZ and DBAC), it was not always the case that the cell wall permeability increased accordingly. For example, solar exposure did not result in a significant increase in propidium iodide (PI) fluorescence, indicating no changes in cell permeability compared with the control (Fig. 3a). Instead, increased fluorescence was observed in cell cultures treated with CMZ or DBAC. Fluorescence increased by 8.8- and 8.6-fold

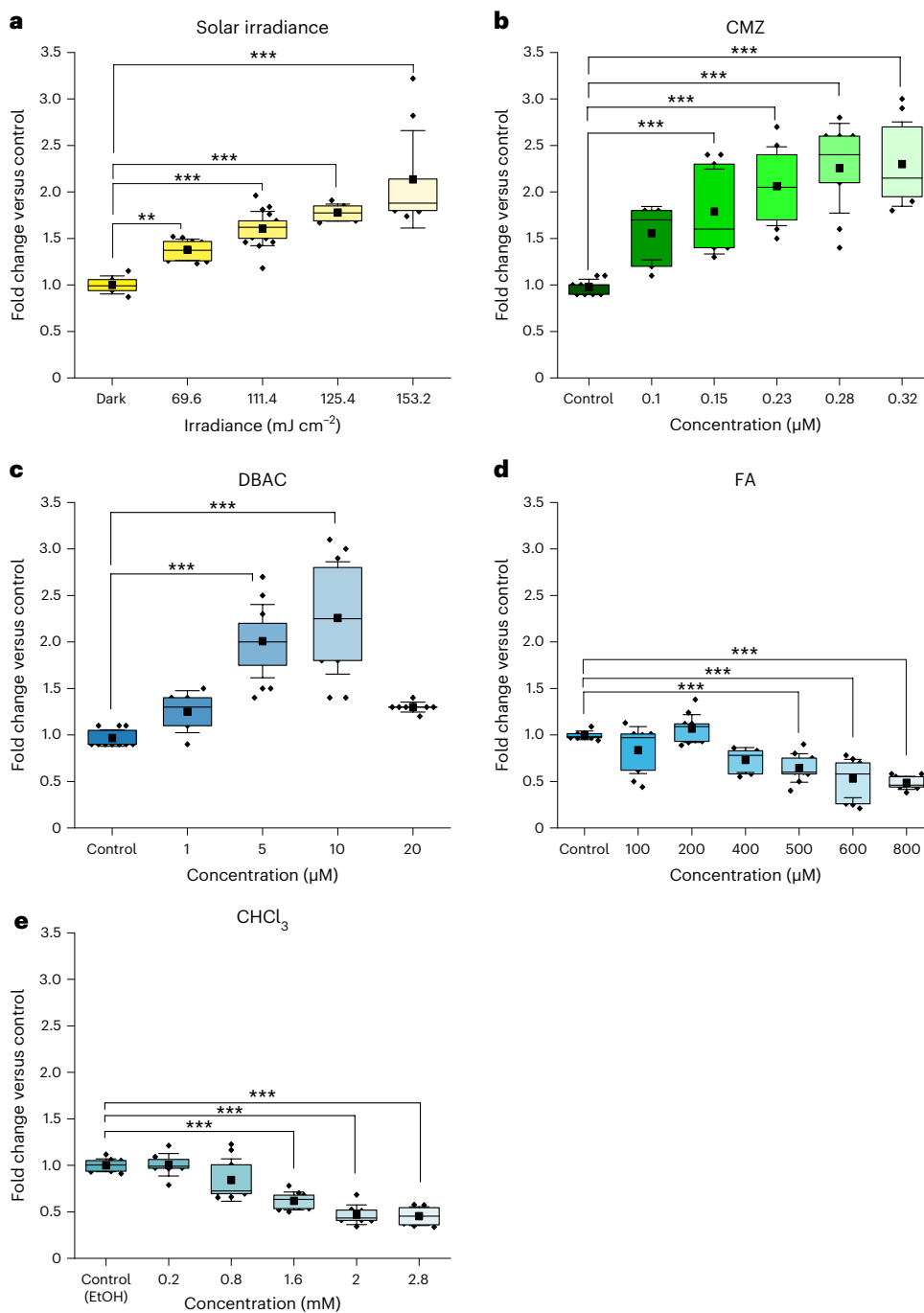


Fig. 1 | The individual impact of factors on natural transformation in *A. baylyi* ADP1 reporter strain. a–e. Fold change of transformation frequency of treated cells in the varying concentration of solar irradiance (a), CMZ (b), DBAC (c), FA (d) and CHCl_3 (e). Solar results were adapted from a previous study conducted by our group¹⁴. All experiments were conducted in three independent biological replicates and three technical replicates per experiment, as indicated by data points (outliers are not included). All box charts represent the 25th and 75th

percentile. The bottom of the box is the first quartile (25th percentile), and the top of the box is the third quartile (75th percentile). The line in the middle of the box is the median (50th percentile). The black squares represent the mean. The whiskers represent the s.d. Significant differences between different treated groups were analysed using ANOVA by the Holm–Bonferroni method for multiple comparisons. Not all significant differences were shown to facilitate clear visuals. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. All P values are listed in (Supplementary Table 1).

for cells treated with CMZ and DBAC, respectively, and was significantly higher than that of the stained controls ($P < 0.01$, Holm–Bonferroni method, Fig. 3a). We further analysed the impact of CHCl_3 and FA on cell membrane permeability as both stressors have been reported previously to alter cell membrane^{29,30}. However, cells treated with FA and CHCl_3 did not display an increase in fluorescence (Fig. 3a) at the concentrations tested here.

ROS production can facilitate natural transformation

As the production of ROS has been associated with an increase in natural transformation frequency in earlier studies^{14–17}, we tested the ability of the selected stressors to elicit the formation of ROS. Since ROS production due to solar irradiance exposure has been reported in previous literature^{14,31}, we analysed the ability of the remaining stressors (that is,

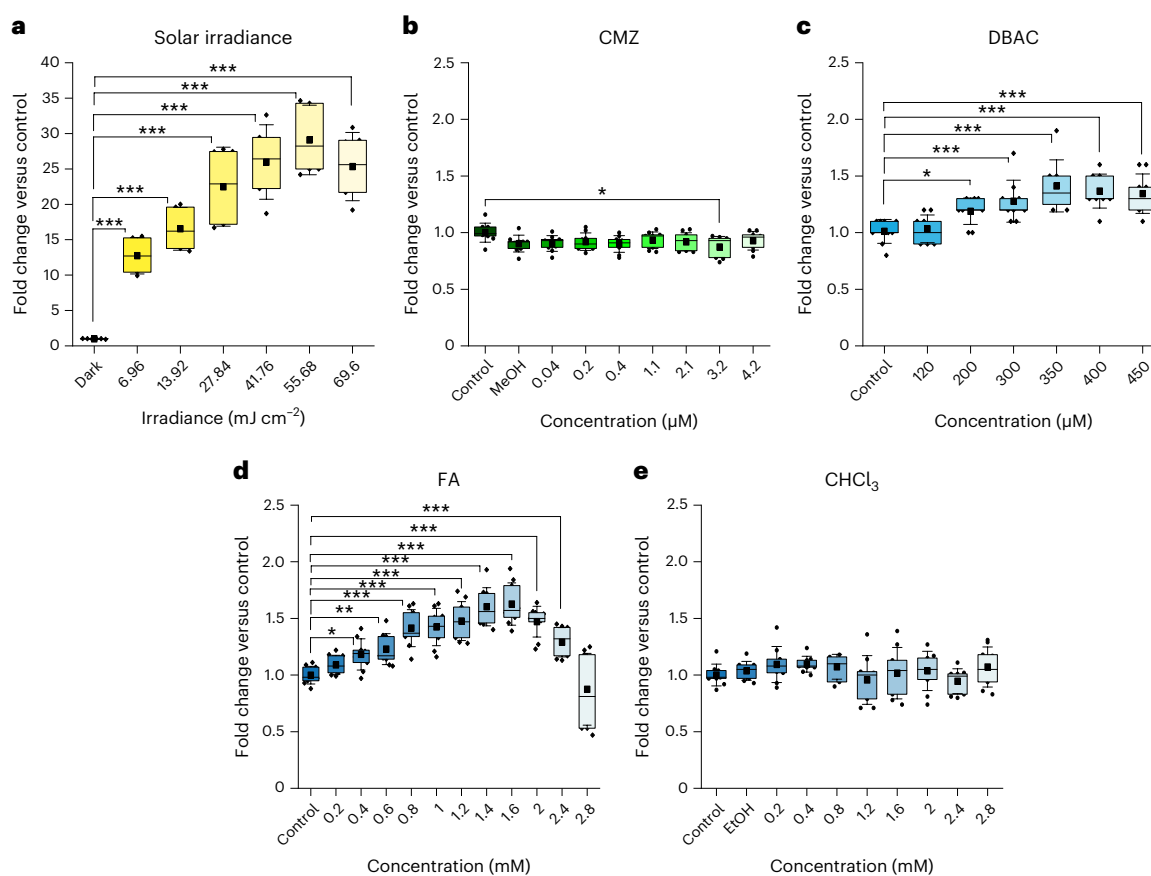


Fig. 2 | Mutagenicity level of the different stressors expressed in terms of the fold change of *Salmonella enterica* Typhimurium TA100 revertant cell numbers compared with control. a–e, Stressors tested include solar irradiance (a), CMZ (b), DBAC (c), FA (d) and CHCl₃ (e). All experiments were conducted in three independent biological replicates and three technical replicates per experiment as indicated by data points (outliers are not included). All box charts represent the 25th and 75th percentile. The bottom of the box is the first quartile

(25th percentile) and the top of the box is the third quartile (75th percentile). The line in the middle of the box is the median (50th percentile). The black squares represent the mean. The whiskers represent the s.d. Significant differences between different treated groups were analysed using ANOVA by the Holm–Bonferroni method for multiple comparisons. Not all significant differences were shown to facilitate clear visuals. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. All P values are listed in (Supplementary Table 2).

CMZ, DBAC, FA and CHCl₃) to enhance ROS production in *A. baylyi* ADP1. Among these, CMZ-, DBAC- and CHCl₃-treated *A. baylyi* cultures exhibited increased ROS production. This was determined by a 1.3-, 1.5- and 1.9-fold increase in the fluorescence of the reported dye (Fig. 3b), which was statistically significant compared with that of the stressor-free controls ($P < 0.001$, Holm–Bonferroni method). Increased ROS formation was not observed in cell cultures treated with FA. Instead, the reporter dye fluorescent signal decreased by 0.3-fold when compared with that of the untreated controls.

Direct interaction of environmental stressors with eDNA

Even though FA displayed a mutagenic response in our cellular assays with TA100, transformation rates of FA-treated *A. baylyi* decreased in a concentration-dependent manner. Similarly, while CHCl₃ exposure resulted in a significant increase in ROS production, transformation rates decreased in a concentration-dependent manner. These observations lead us to speculate that the decrease in transformation rates might be associated with damage to the eDNA during chemical treatments, as both of these chemicals have been reported to cause damage by chemically interacting with the DNA molecule^{29,30,32}. To evaluate this, we pre-treated the eDNA for 24 h with either FA or CHCl₃ (500 μM and 1.6 mM, respectively) and dosed the pre-treated DNA with stressor-free *A. baylyi* transformation assays. DNA pre-treatment led to a significant decrease in transformation rates in our stressor-free assays (Fig. 3c).

This suggests that the negative impact on transformation rates previously observed (Fig. 1d,e) is the result of direct damage to the donor DNA that may result in DNA fragments with non-functional promoter information or fragments with structural damage that were not able to integrate into the bacterial chromosome.

Impact of paired stressors on natural transformation outcome

We hypothesize that, when environmental stressors—which elicit natural transformation—are co-incubated with *A. baylyi*, the transformation outcome of these co-incubation assays will be consistent with the geometric addition of the individual effect on the transformation of each individual stressor. To evaluate this, the response values from the single-agent assays were statistically compared with the combined agents' response values. By definition, four types of response are expected and catalogued as: (1) additive if the mean of the observed response is not statistically different from the mean of the calculated geometric addition; (2) synergistic, if the mean of the observed response results in a significant increase in transformation fold change compared with both stressors and that the observed response is significantly higher than geometric addition; (3) neutral, if the mean of the observed response results in an insignificant difference compared with the transformation of the single stronger stressor (defined as the stressor that is inducing the comparatively higher transformation outcome of the pair); and (4) antagonistic, if the mean of the observed

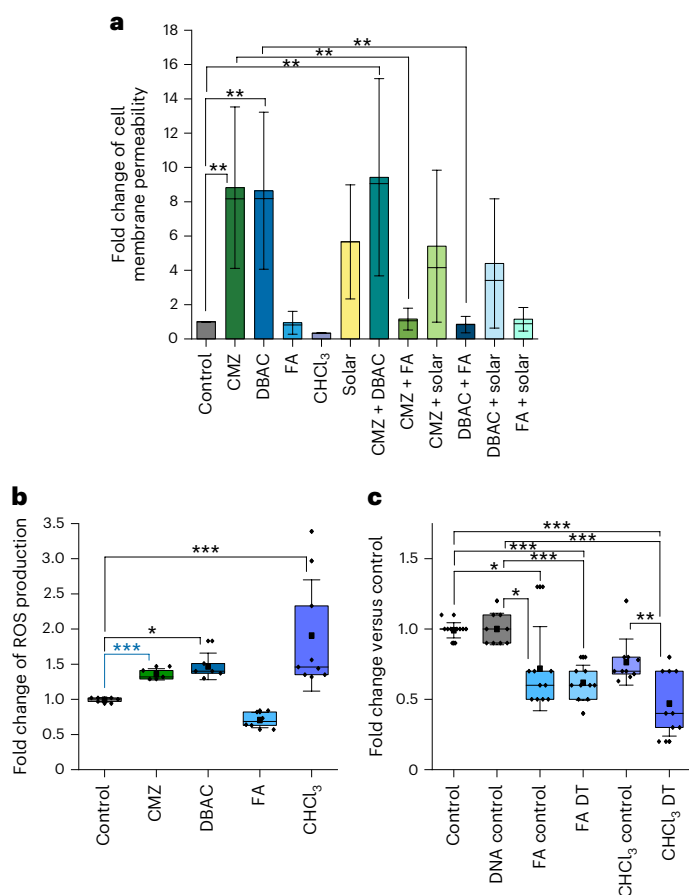


Fig. 3 | Effect of various stressors. a, Fold change of stained cells with PI, to analyse cell permeability upon treatment with CMZ (0.28 μM), DBAC (5 μM), solar (41.76 mJ cm^{-2}), CHCl_3 (1.6 mM) and FA (500 μM), exposed to *A. baylyi* ADP1, either individually or in combined pairing. **b**, Fold change of ROS production in *A. baylyi* treated with CMZ, DBAC, CHCl_3 and FA individually. **c**, Fold change of transformation frequency of cells provided with donor DNA that was previously treated with 500 μM of FA (FA DT, DT denotes dDNA treated) or 1.6 mM CHCl_3 (CHCl_3 DT, DT denotes dDNA treated). Definitions of controls can be found in Supplementary Methods 1.3. All experiments were conducted in three independent biological replicates and three technical replicates per experiment as indicated by data points (outliers are not included). All box charts represent the 25th and 75th percentile. The bottom of the box is the first quartile (25th percentile), and the top of the box is the third quartile (75th percentile). The line in the middle of the box is the median (50th percentile). The black squares represent the mean. The whiskers represent the s.d. The error bars represent the s.d. Significant differences between different treated groups were analysed using ANOVA by the Holm–Bonferroni method for multiple comparisons (two-sided *t*-test in blue). Not all significant differences are shown to facilitate clear visuals. * $P < 0.05$, ** $P < 0.01$, **** $P < 0.001$. All *P* values are listed in Supplementary Tables 1, 3 and 4.

response falls below significantly compared with the transformation outcome by the single stronger stressor. Significance testing is done as described in Methods.

All four expected transformation outcomes were observed in the co-incubation assays. The stressor interactions evaluated in this study were catalogued as (1) synergistic, (2) additive, (3) antagonistic and (4) neutral. Figure 4 summarizes the fold changes of natural transformation frequency for each paired combination and the comparison between the theoretical and actual outcomes for transformation fold change by combined stressors. All tested permutations did not result in any cytotoxicity compared with the control since the total cell counts were not significantly different from that of controls (Supplementary Fig. 3).

Pairing stressors lead to additive or synergistic responses

Additive interactions were observed once DBAC (5 μM) and solar irradiance (41.8 mJ cm^{-2}) were combined. To illustrate, individual treatment of DBAC and solar resulted in 1.8- and 2-fold change, respectively. The geometric addition range of both factors should result in a 2.3–3.6-fold change, which agrees with the observed outcome when both factors are combined ($P < 0.001$, *t*-test method) (Figs. 4 and 5). The combination of CMZ with solar demonstrated synergistic interaction. When paired, solar (41.8 mJ cm^{-2}) + CMZ (0.28 μM) resulted in a 3.1-fold change relative to control, which is significantly higher than that of its individual effect and the geometric addition range of both factors' 1.8–2.9-fold change ($P < 0.001$, *t*-test method) (Figs. 4 and 5).

Pairing stressors lead to neutral transformation response

Neutral interactions were recorded for three stressor combinations, namely FA + CHCl_3 , CMZ + DBAC and solar irradiance + FA. Since both FA and CHCl_3 do not contribute to natural transformation (Fig. 1d,e), the combination of FA and CHCl_3 resulted in a somewhat neutral response whereby there was an insignificant difference in the outcome of natural transformation compared with either of the stressors and significantly different from the expected geometric range ($P < 0.01$, *t*-test method) (Figs. 4 and 5b). Such neutral response was also observed for the pairing of CMZ and DBAC. Both CMZ and DBAC resulted in an increase in natural transformation frequency (Fig. 1b,c). However, the combination of CMZ and DBAC resulted in an insignificant increase in transformation, at 2.1-fold, compared with individual treatments, and a significant difference from the expected geometric range ($P < 0.01$, *t*-test method) (Figs. 4 and 5b). The outcome of this pairing is not associated with the cytotoxicity effect as the total population number of treated and non-treated cells remained the same (Supplementary Fig. 3e). Lastly, when FA was paired with solar (a strong inducer of natural transformation frequency, Fig. 1a), FA did not significantly affect the final outcome of the natural transformation in a detrimental manner (Figs. 4 and 5b).

Pairing stressors lead to antagonistic response

Antagonistic interactions were recorded for five interactions, particularly when pairing FA or CHCl_3 with other stressors. To illustrate, when FA was individually paired with CMZ, the additive increment was no longer observed. Instead, the combination of CMZ with FA resulted in a significant decrease in the natural transformation that is similar to the individual treatment of FA. Pairing FA with DBAC also reverted the increase in the natural transformation that was observed with individual treatment of DBAC, which decreased from 1.8-fold for DBAC alone to baseline levels in co-incubation assays ($P < 0.001$, *t*-test method, Figs. 4 and 5c).

Antagonistic interactions were also observed with the combination of CHCl_3 with solar, CMZ and DBAC (Fig. 5c). To illustrate, the individual treatment of solar, CMZ and DBAC resulted in 2-, 1.8- and 1.8-fold enhancement in natural transformation frequency, respectively. However, when each of these stressors was combined with CHCl_3 , the natural transformation frequency reverted to almost baseline levels at 1.3-, 1.0-, and 1.1-fold change, respectively ($P < 0.001$, *t*-test method, Figs. 4 and 5c).

Gene expression profiles

We examined the expression profiles of two genes involved in DNA translocation (that is, *pilX* and *comA*) and three genes associated with DNA repair (that is, *uvrB*, *recA* and *ddrR*). These genes were selected as (1) they have been reported to show increased expression in cell cultures displaying enhanced transformation frequencies upon exposure to solar irradiance¹⁴ and bromoacetic acid¹⁵, and (2) transcript numbers for these genes increased in a concentration-dependent manner when exposed to the stressor condition. This evidence suggests a strong

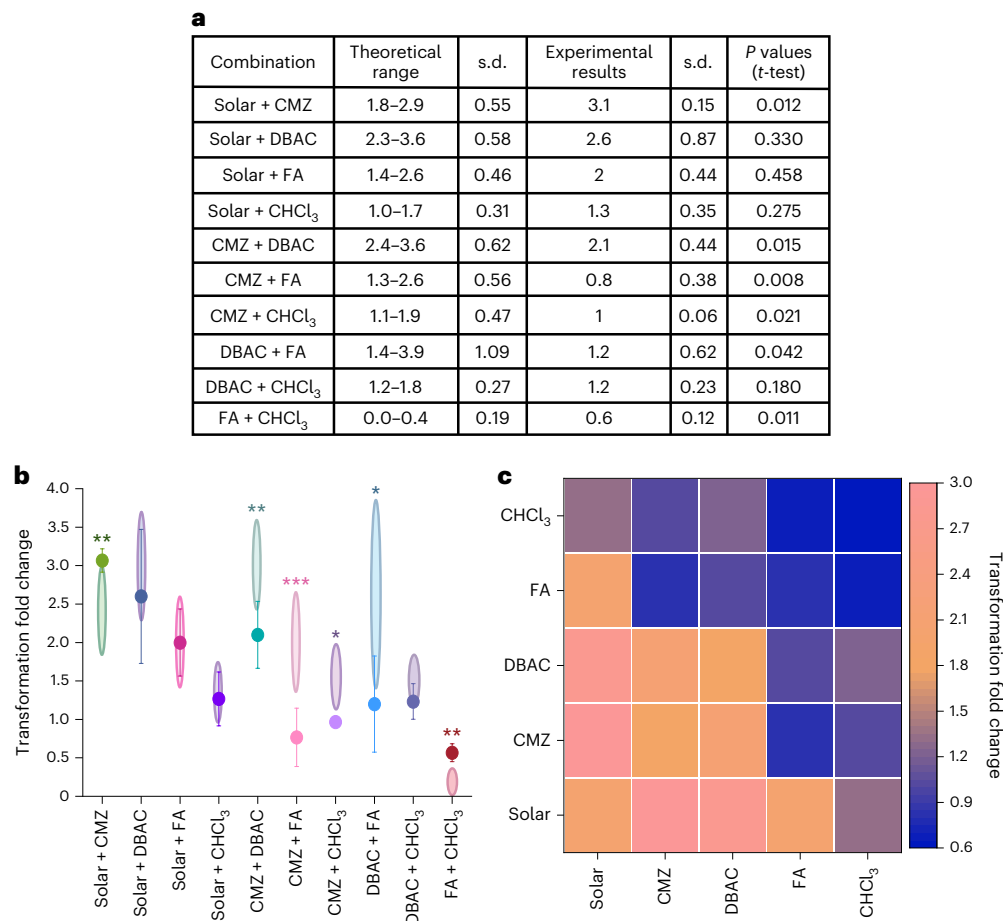


Fig. 4 | Comparison between the calculated theoretical range and wet-bench experimental results, and summary chart of combined results.

a, Calculated theoretical range using the Bliss equation compared with wet-bench experimental results. s.d. and *P* values from the *t*-test are included to denote differences in the two sets of results. **b**, A demonstration of the comparison between the calculated theoretical range (represented in cloud form) and wet-bench experimental results (represented in circle form). The calculated values were done from data obtained from three independent biological replicates and three technical replicates per experiment (outliers were

not included). The circle represents the mean. The error bars represent the s.d. Significant differences between different treated groups were analysed using the one-sided *t*-test method. **P* < 0.05, ***P* < 0.01, ****P* < 0.001. **c**, A summary chart of combined transformation assays where pink colour represents the highest observed transformation fold change, orange or purplish orange indicates an increase in transformation frequency, and varying intensities of blue indicate a neutral or decrease in transformation frequency. All experiments were conducted in three independent biological replicates and three technical replicates per experiment. All *P* values are listed in Supplementary Table 1.

relationship between the transformation enhancement phenotype and the expression of the selected genes. In this study, expression profiles for DNA translocation genes were used as a proxy for effects on cell permeability, while expression profiles for DNA repair genes were used as a proxy for DNA damage response.

Expression profiles were consistent with the stressor's mode of action. Stressors that displayed a mutagenic response in TA100 or ROS formation (that is, solar irradiance, DBAC, FA, DBAC and CHCl₃) showed increased expression of DNA damage response genes (*P* < 0.05, *P* < 0.01 and *P* < 0.001, *t*-test method, Fig. 6). To illustrate, FA induces mainly DNA crosslinking, a type of DNA damage repaired by nucleotide excision²⁸. Consequently, *A. baylyi* cell cultures exposed to FA showed a significant increase in the expression of *uvrB* (*P* < 0.01, *t*-test method, Fig. 6d), a central enzyme in nucleotide excision repair³³. Similarly, transcript numbers for *pilX* and *comA* significantly increased in CMZ and DBAC treatments (*P* < 0.05, *P* < 0.01 and *P* < 0.001, *t*-test method, Fig. 6b,c), which previously showed cell permeability effects in *A. baylyi* cell cultures (Fig. 3a).

Even though *recA* was not overexpressed in additive and synergistic stressor combinations, DBAC and CMZ combinations with solar irradiance led to significantly higher expression of *uvrB* (CMZ + solar,

fivefold change; DBAC + solar, fivefold change, *P* < 0.05 and *P* < 0.001, respectively, *t*-test method, Fig. 6a) when compared with solar single treatment where only twofold change increase was observed. Similarly, *ddrR* expression was upregulated from threefold change by single treatment of solar to up to 8.3-fold for CMZ + solar and fivefold change for DBAC + solar (*P* < 0.001, *t*-test method, Fig. 6a). These results suggest a stronger DNA damage and repair response in solar irradiance combinations, which was previously associated with increased transformation frequencies^{14,15}.

Gene expression profiles in response to antagonistic stressor combinations can be grouped into two subclasses. The first subclass corresponds to expression profiles displaying an overall decrease in transcripts for DNA translocation and DNA damage and repair genes compared with the control or the single treatment (that is, solar + CHCl₃, CMZ + CHCl₃, DBAC + CHCl₃, DBAC + FA) (*P* < 0.05, *P* < 0.01 and *P* < 0.001, *t*-test method, Fig. 6). Decreased expression of these genes is consistent with the antagonistic response. We speculate that the presence of CHCl₃ and FA—which are known to directly interact with protein complexes^{34–36}—may impair the ability of the cells to sense damage and elicit a transcriptional response. The second subclass of expression profiles corresponds to antagonistic combinations

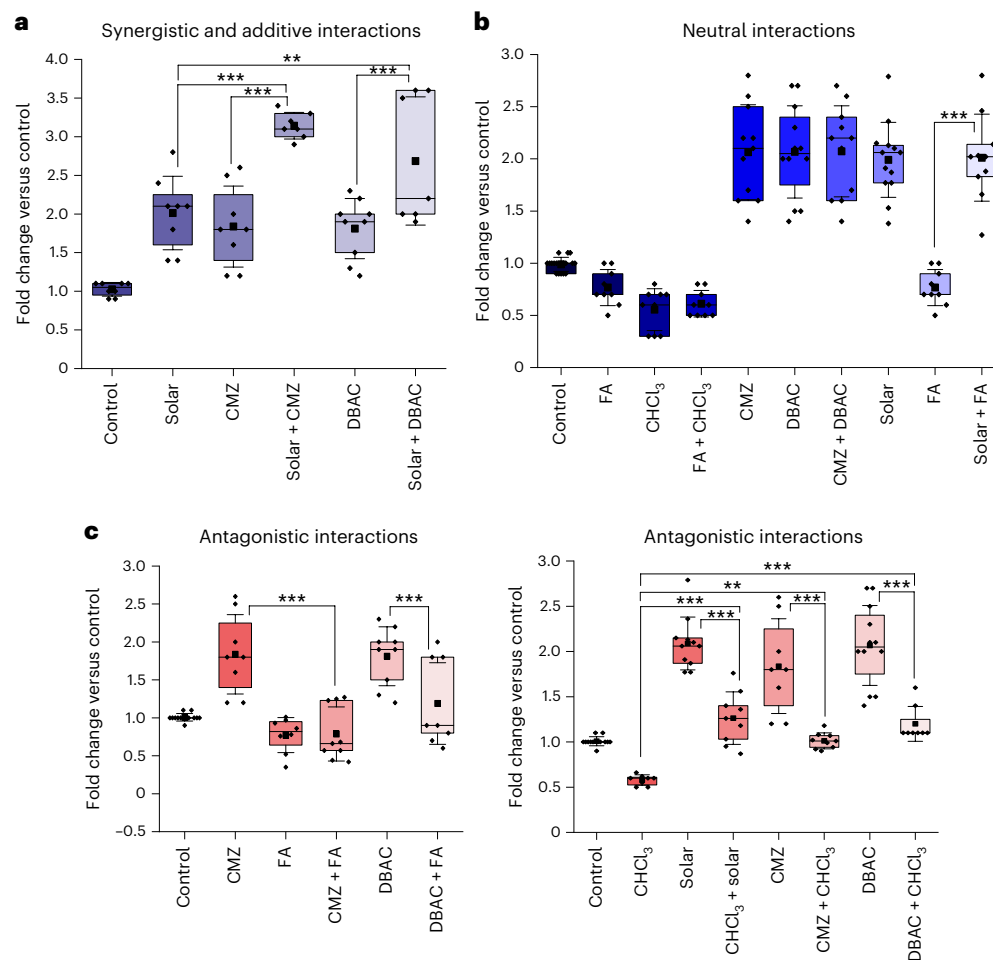


Fig. 5 | Fold changes of natural transformation frequency observed in *A. baylyi* ADP1 when exposed to paired combinations of stressors. a–c, The represented impact per stressor was observed at the following concentrations, solar (41.76 mJ cm^{-2}) CMZ ($0.28 \text{ }\mu\text{M}$), DBAC ($5 \text{ }\mu\text{M}$), FA ($500 \text{ }\mu\text{M}$) and CHCl_3 (1.6 mM). The outcome is categorized into four categories, namely synergistic and additive interaction, which is defined as a higher natural transformation frequency when the stressors were paired compared with that obtained by the stressor alone (a); neutral interaction, which is defined when the combination of two stressors results with an insignificant response compared with one or both of the individual treatment (b); and antagonistic interaction, which is defined when the combination of two stressors results in a significant decrease in natural

transformation when compared with either of the individual treatments (c). All experiments were conducted in three independent biological replicates and three technical replicates per experiment as indicated by data points (outliers are not included). All box charts represent the 25th and 75th percentile. The bottom of the box is the first quartile (25th percentile), and the top of the box is the third quartile (75th percentile). The line in the middle of the box is the median (50th percentile). The black squares represent the mean. The whiskers represent the s.d. Significant differences between different treated groups were analysed using ANOVA by the Holm–Bonferroni method for multiple comparisons. Not all significant differences are shown to facilitate clear visuals. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. All P values are listed in Supplementary Table 1.

displaying an increased expression of DNA translocation and repair genes (that is, FA + CMZ) (Fig. 6). For the FA + CMZ combination, the increased expression profiles are inconsistent with our previous hypothesis, and a closer look into the molecular regulation for this stressor combination is worth exploring.

Gene expression profiles corresponding to neutral interaction displayed unpredictable expression compared with transformation outcomes (Figs. 4, 5b and 6b). To elucidate, the gene expression profile of combined CMZ and DBAC upregulated the expression of *piIX* (DNA translocation), with a 5.2-fold change compared with the single treatments (2.8- and 2.2-fold change, respectively) and does not support the observed neutral response ($P < 0.01$, t -test method, Fig. 6b). Moreover, even though FA and CHCl_3 combination resulted in a neutral outcome, there was an upregulation in the *ddrR* gene compared with their respective individual stressor ($P < 0.001$, t -test method, Fig. 6d,e). In contrast, the neutral outcome is supported by the gene expression results where the presence of FA with solar irradiance downregulated the expression of competency genes ($P < 0.05$, $P < 0.01$ and $P < 0.001$, t -test method,

Fig. 6a,d) but the expression of DNA integration genes such as *recA* and *ddrR* is still very similar between solar + FA and solar (Fig. 6a,d).

Despite treatment-to-treatment variations, overall, stressor combinations displaying additive and synergistic transformation outcomes lead to increased expression of both gene categories (that is, DNA translocation and DNA repair) compared with single-stressor treatments. Gene expression profiles associated with antagonistic stressor combinations, in general, are associated with decreased expressions of both gene categories, while neutral stressor combinations arise from more variable gene expression profiles.

Natural transformation outcomes in environmental samples

To verify the variability in natural transformation outcomes of samples with multiple stressors, we exposed *A. baylyi* ADP1 to chlorinated water samples that were collected from a full-scale wastewater treatment process (WWTP) that was stably operated (Supplementary Fig. 4a) but exhibited a downward trend in the turbidity and free residual chlorine

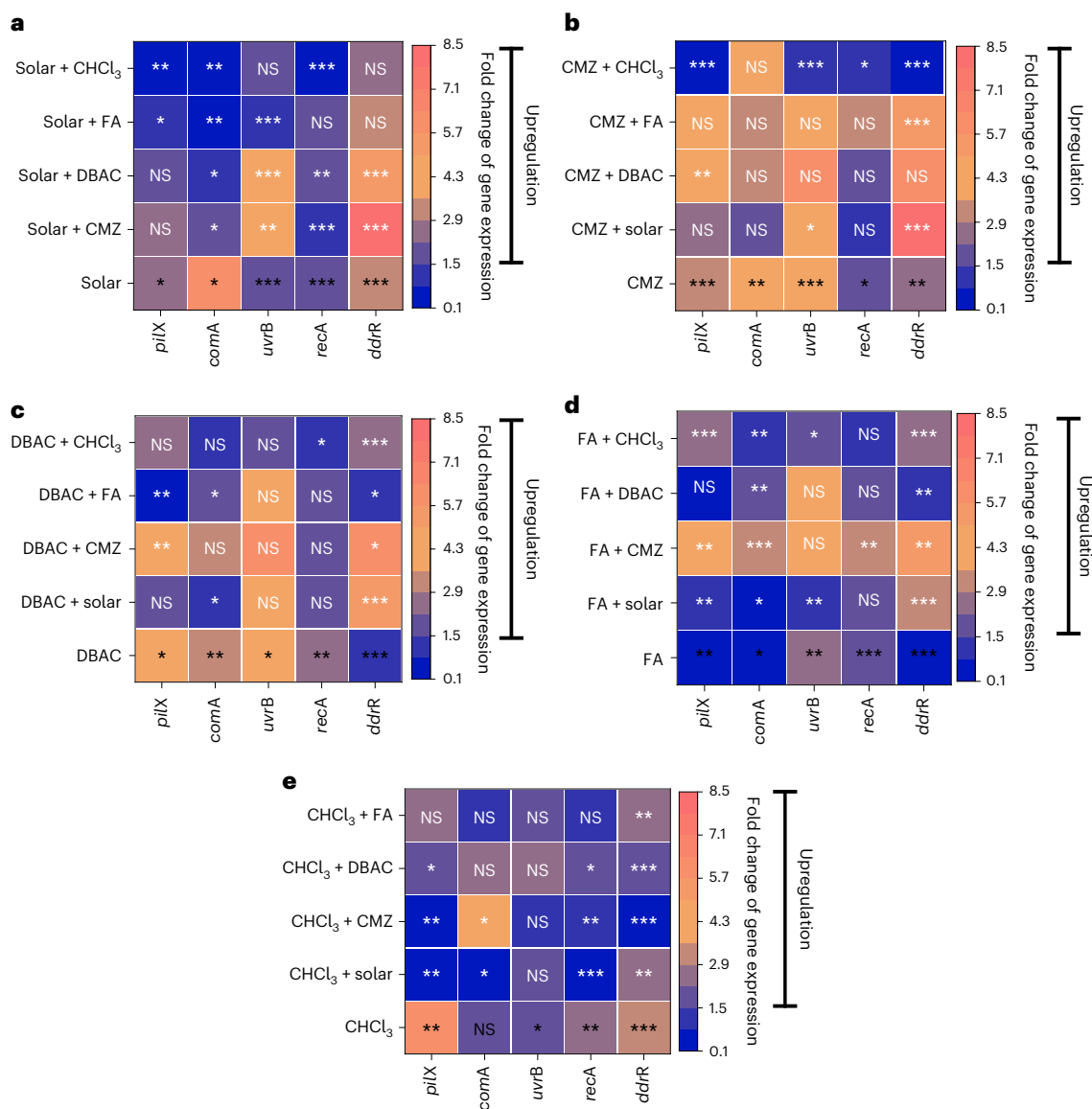


Fig. 6 | Fold changes of expression of genes involved in both DNA translocation and integration at 60 min post-treatments with single and multiple stressors. a–e, The represented impact per stressor was observed at the following concentrations, solar (41.76 mJ cm⁻²), CMZ (0.28 μM), DBAC (5 μM), FA (500 μM) and CHCl₃ (1.6 mM). Pink, orange and purplish orange indicate upregulation (>1.4). Blue and dark blue indicate neutral impact or downregulation (<1.4). **a,** Solar irradiance when individually treated and when combined with other stressors. **b,** CMZ when individually treated and when combined with other stressors. **c,** DBAC when individually treated and when

combined with other stressors. **d,** FA when individually treated and when combined with other stressors. **e,** CHCl₃ when individually treated and when combined. All experiments were conducted in three independent biological replicates and two technical replicates per experiment. Significant differences between different treated groups were analysed using the two-sided *t*-test method. **P* < 0.05, ***P* < 0.01, ****P* < 0.001. NS, not significant. White labels represent the differences between single-treated samples and multiple-treated samples. Black labels represent the differences between single-treated samples and control samples. All *P* values are listed in Supplementary Table 5.

concentration in the sampled treated wastewater (Supplementary Fig. 4b). The samples were further characterized for their suite of chemical compounds (Fig. 7a and Supplementary Fig. 5). It was observed that 19.1–44.9% of the total characterized chemicals were drugs and 40.6–59.5% of the total characterized chemicals were medications. Despite similarities in the chemical composition and stable performance in the WWTP during that short sampling duration, the chlorinated effluent resulted in different extents of natural transformation. For example, samples that were collected on 5 October, 26 October and 7 November increased transformation by 1.6-, 2.4- and 2.1-fold, respectively, compared with the control (*P* < 0.01, Holm–Bonferroni method). In contrast, samples that were collected on 11 October decreased transformation to 0.5-fold compared with control (*P* < 0.001,

Holm–Bonferroni method). The remaining samples did not show a significant change in the natural transformation frequency compared with controls (Fig. 7b). There was no apparent correlation between these natural transformation outcomes and the downward trend of free residual chlorine concentration present in the samples.

Minimal amount of eDNA to mitigate natural transformation

Considering that natural transformation outcomes are hard to predict and highly variable, we aim to determine how the impact of defined positive factors for natural transformation can be mitigated by limiting the incidence of donor DNA (that is, eDNA). We performed experiments where all samples are exposed to solar irradiance (41.8 mJ cm⁻²) and

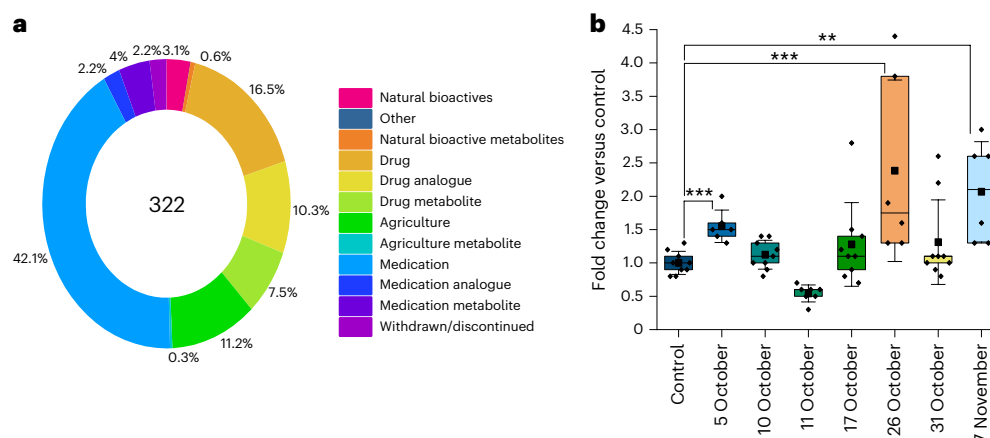


Fig. 7 | Characterization of chlorinated effluent samples and their contribution towards natural transformation. a, An average profile of chemicals identified in the chlorinated effluent. Chlorinated effluent samples were collected over a short time frame from 5 October 2021 to 7 November 2021. The wastewater treatment plant is performing stably during this period. **b,** Natural transformation frequencies were obtained by each chlorinated effluent sample. All experiments were conducted in three independent biological replicates and three technical replicates per experiment as indicated by data

points (outliers are not included). All box charts represent the 25th and 75th percentile. The bottom of the box is the first quartile (25th percentile) and the top of the box is the third quartile (75th percentile). The line in the middle of the box is the median (50th percentile). The black squares represent the mean. The whiskers represent the s.d. Significant differences between different treated groups were analysed using ANOVA by the Holm–Bonferroni method for multiple comparisons. Not all significant differences were shown to facilitate clear visuals. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. All P values are listed in (Supplementary Table 1).

treated with DBAC (5 μM). This pairing of stressors was shown earlier to highly increase natural transformation frequencies (Figs. 4 and 5a). When supplemented with 4 and 2 $\mu\text{g ml}^{-1}$ of donor DNA, transformation increased by 3.1- and 2.6-fold, respectively, compared with the control. The natural transformation frequency decreased as donor DNA concentration decreased. Specifically, the natural transformation frequency was similar to that of control when 0.1 $\mu\text{g ml}^{-1}$ of donor DNA was added to 2×10^7 (cell density), and this natural transformation frequency further decreased to 0.6-fold when *A. baylyi* ADPI cells were supplemented with 0.05 $\mu\text{g ml}^{-1}$ of donor DNA (Supplementary Fig. 6).

Discussion

Increasing water scarcity in many countries has prompted a renewed emphasis on the need to reuse treated wastewater. While WWTPs serve as important barriers to remove majority of the contaminants before reuse, the current technologies do not achieve high log removal efficiency of eDNA and organic micropollutants^{4,6,37–39}. Furthermore, the use of chemical-based disinfectants such as chlorine can result in the formation of toxic DBPs^{40–42}. The confluence of eDNA, along with sublethal concentrations of organic micropollutants and DBPs, can create a suitable environment for enhanced horizontal gene transfer. This study hypothesized that upon exposure to cumulative types of stressor that are either mutagenic, produce ROS and/or affect cell membrane permeability, a bacterial host would trigger a response to repair the damage and thereafter result in a corresponding additive outcome in natural transformation frequency. However, our findings suggest that the hypothesized additive outcome was not always observed. Instead, synergistic, antagonistic or neutral outcomes were evidenced.

Despite our nullified hypothesis, we wanted to determine if there exists a pattern that would allow us to predict the natural transformation outcomes arising from the co-presence and interaction of stressors. We elucidated the mechanisms of each stressor on natural transformation which is summarized in Supplementary Fig. 7. First, we observed that when there are two stressors, with one of them mainly contributing to enhancing cell wall permeability (that is, translocation) and the other one mainly contributing to mutagenicity (that is, which induces DNA damage and triggers DNA repair), a synergistic outcome on natural transformation is obtained. For example, this happened

when solar irradiation (which is more mutagenic than CMZ and DBAC, Fig. 2a) was paired along with DBAC or CMZ (which increase cell membrane permeability, Fig. 3a, but are not as mutagenic compared with solar irradiation). This suggests that the pairing of stressors, each with mechanisms that can positively accelerate both steps of natural transformation (translocation and homologous recombination), will probably lead to the synergistic outcome of natural transformation.

Second, we observed that when both stressors are mainly contributing to enhancing cell wall permeability (for example CMZ paired with DBAC) (Figs. 3a and 6), a neutral response was observed instead (Figs. 4 and 5b). By comparing the gene expression data, we saw that when all genes associated with both competence and DNA repair mechanisms were upregulated at the same timepoint as that for CMZ + DBAC ($P < 0.05$, $P < 0.01$ and $P < 0.001$, t -test method, Fig. 6b,c), a neutral response was observed. This is in contrast to the synergistic natural transformation, which was associated mainly with the upregulation of genes associated with the DNA repair mechanisms at the 60 min exposure timepoint. This difference suggests that the sequential timing of when translocation and integration take place is perhaps more important to facilitate synergistic outcomes of natural transformation than having all genes upregulated simultaneously at the same time. Alternatively, facilitating DNA integration by triggering DNA repair probably imposes a relatively more positive outcome on natural transformation than that of translocation. An earlier study showed that when using a chromosomal DNA of about 2,000 bp as donor DNA, only 0.1% of the total *Acinetobacter calcoaceticus* is transformed successfully^{43,44}. This low natural transformation frequency was due to inefficient integration arising from the strong competition with DNA degradation within the cytoplasm^{43,45}. Hence, it is likely that, by facilitating DNA repair, the integration of DNA happens faster than the rate of DNA degradation, and thus resulted in more donor DNA being integrated into the chromosome.

Third, we observed that, when one of the stressors is able to interact directly with the eDNA, its effect outweighs that of the other stressor. For example, pairing either FA or CHCl_3 with most of the other tested stressors generally cancelled out any increase in natural transformation brought upon by the other stressor and resulted in an antagonistic response. FA and CHCl_3 are traditionally used as

preservatives. FA can react with thiol and amine groups in both DNA and protein in the cell, and result in either DNA–DNA, protein–protein or DNA–protein cross-link³⁰. Likewise, CHCl₃ when dissolved in ethanol, is used as a fixative which can thus affect DNA^{29,32}. Moreover, CHCl₃ can disturb the membrane bilayer causing cell lysis and release of its content⁴⁶, which potentially can affect the DNA translocation step of natural transformation. However, our findings suggest that there are no significant changes compared with control in cell wall permeability when cells were treated with FA or CHCl₃ (Fig. 3a). Instead, when purified donor DNA was treated with FA or CHCl₃ first before introduction to *A. baylyi* that are not exposed to either of the chemical stressors, a significant decrease in natural transformation frequency compared with the respective controls was observed (Fig. 3c). This suggests that a direct interaction between donor DNA and both chemicals inhibited subsequent integration of the DNA into the chromosomal genome. Collectively, our findings suggest that, while the mechanisms behind the induction of natural transformation are accounted for, interactions between stressors and the eDNA exist. Such interactions may cause the overall outcome to deviate from the geometric additive outcome of individual responses, in turn making it hard to predict what the end natural transformation outcome may be.

The challenge of forming a prediction on the natural transformation outcome is further exacerbated by the fact that wastewater contains a myriad of chemical contaminants. An earlier attempt to in silico characterize these chemical contaminants has shown that many of the chemicals present in untreated wastewater remain poorly characterized⁴⁷. In this study, natural transformation experiments were performed on chlorinated effluent from a WWTP that was stably operated (Supplementary Fig. 4a). Despite this, chemical profiles still differed slightly among the samples (Supplementary Fig. 5 and Supplementary Table 6), albeit differences in the chemical profiles did not correlate with the natural transformation outcomes. While there was positive induction in natural transformation frequency compared with control for some of the chlorinated effluent samples, which was similar to earlier observations^{15,18}, it was in a rather ad hoc manner. The variable nature of responses from the chlorinated effluent is in agreement with the likewise variable nature of responses observed from our individual and combined stressor experiments, where highly complex chemical mixtures behave non-linearly and make it difficult to predict the final outcome.

While it is difficult to characterize the outcome arising from all stressors present in treated effluent, further lowering the eDNA concentration to <0.1 ng l⁻¹ per 2 × 10⁷ competent cells was shown to lower natural transformation frequency back to control level despite being subjected to the pairing of stressors that were strongly inducing natural transformation. An earlier study reported 8.6 μg l⁻¹ of eDNA in tertiary-treated wastewater⁴, and further coupling wastewater treatment processes with membrane filtration can possibly further reduce extracellular genes by 2- to 4-log, depending on the fouling extent of the membrane filtration unit⁴⁸. However, even with such additional membrane filtration, the eDNA concentration may still be higher than 0.1 ng l⁻¹ based on the initial 8.6 μg l⁻¹ of eDNA reported by Calderon-Franco et al.⁴. Alternatively, inactivating the eDNA by means of ultraviolet light such that they are no longer functional upon DNA integration can potentially also alleviate natural transformation¹⁴.

To conclude, instead of a geometric addition, our study found different interactions outcome for natural transformation when stressors co-exist. Specifically, outcomes can deviate from geometric addition and instead be synergistic, neutral or antagonistic. Moreover, we demonstrated the difficulty to predict natural transformation outcomes in more complex settings, irrespective of the prior knowledge associated with the stressors. As such, we recommend to act on the basis of precautionary principle and to invest in wastewater treatment processes that minimize concentrations of eDNA and competent bacteria host (for example, membrane filtration-based processes) as the key intervention

strategy in minimizing natural transformation frequency arising from the use of treated wastewater.

Methods

Bacterial strains

To determine natural transformation frequency, a reporter strain of *A. baylyi* ADPI cells^{14,15,49} was used. The reporter strain is genetically modified to carry a marker-reporter rescue cassette (*rbcl*-Δ*P aadA*::*GFP*) integrated into the bacterial chromosome between the *lipB* and *lipA* genes. The gene fusion (*aadA*::*GFP*) encoding spectinomycin resistance (*aadA*) and green fluorescent protein (GFP) lacks a functional promoter and is therefore not expressed in the recipient strain. To restore the expression of *aadA*::*GFP*, donor DNA carrying a constitutive functional promoter (*rbcl*-*P_{rm}*-*aadA*) is used. The donor DNA, therefore, represents the eDNA in this study. Upon the uptake of the donor DNA/eDNA through translocation, homologous recombination subsequently takes place between the flanking *rbcl* and *aadA* loci, leading to the insertion of the *P_{rm}* promoter upstream of *aadA*::*GFP*. The integration of the promoter activates the transcription of the gene fusion, restoring spectinomycin resistance and fluorescence. This strain was provided by Prof. Daniele Daffonchio's group at King Abdullah University of Science and Technology (KAUST). To determine mutagenicity, *Salmonella enterica* Typhimurium TA100 was used^{15,26}. TA100 has pre-existing mutations that leave the bacteria unable to synthesize the required amino acid histidine and therefore unable to grow in its absence. When new mutations, specifically in the form of base pair substitution mutation for TA100, occur at the site of the pre-existing mutation or nearby in the genes, the gene's function can be restored to allow the cells to synthesize histidine and grow on minimal agar media. The tester strain TA100 has been genetically modified to increase cell permeability and repair mechanisms²⁶, and therefore, mutagenic and cytotoxic thresholds observed in TA100 cannot be directly extrapolated to other bacteria. However, the TA system remains the current standard assay to determine the mutagenicity of compounds against bacteria²⁶. This strain was provided by Michael Plewa's group at the University of Illinois Urbana-Champaign (UIUC).

Types of stressors

A total of five types of stressor, namely solar irradiation (solar) representing an environmental stressor, pharmaceutical compound CMZ and disinfection byproducts such as DBAC, FA and CHCl₃, were evaluated. Each stressor has a different mechanism of impact on natural transformation; the analyses of the combined effect of these stressors can draw a simple representation of the complexity that is present in the actual environment. Solar irradiation experiments were conducted using an Atlas Suntest XLS+ photo simulator (Ametek) equipped with a xenon arc lamp. The solar simulator was programmed to only emit light with wavelengths between 280 nm and 800 nm at an irradiance of 27.9 mJ h⁻¹. A filter that blocks wavelengths <280 nm (that is, germicidal ultraviolet-C range) was placed above each reaction beaker. The solar irradiance used in this study has a profile that approximated the direct solar irradiance profile measured at KAUST^{31,50}. CMZ was prepared in a stock solution of 5 mg ml⁻¹ (w/v) in methanol, then a series of dilutions were made in potassium phosphate buffer, PPB (for example, dilution A: 1 mg ml⁻¹ in PPB, dilution B: 0.1 μg ml⁻¹ in PPB, and dilution C: 0.01 μg ml⁻¹ in PPB). According to the required final concentration per reaction, the corresponding stock dilution was used. Since methanol concentration became highly diluted with PPB, no methanol control was needed in subsequent experiments. DBAC was prepared in 1 M stock diluted with PPB to obtain the working concentrations needed for experiments. FA was diluted in PPB to obtain a stock of 1 M. CHCl₃ was diluted with ethanol to obtain 1 M stock. As CHCl₃ is soluble only in ethanol, and ethanol can be toxic for the cells, ethanol controls in concentrations similar to that of the CHCl₃ test reactions were also included to ensure observations made were due to CHCl₃ and not

ethanol. All catalogue numbers of chemicals and buffer compositions used in this study are listed in Supplementary Information (Supplementary Tables 2.1 and 2.2).

Natural transformation experiments

Donor DNA preparation. Donor DNA was amplified via polymerase chain reaction (PCR) using TAKARA Ex-Taq polymerase and plasmid template that carries the complete sequence of donor DNA for transformation assay⁴⁹. Donor DNA derived from successful amplification was then purified using Wizard SV Gel and PCR Clean-Up System (Promega) using protocols as described by the manufacturer. The concentration of purified DNA was measured using Qubit dsDNA BR Assay (Thermo Fisher Scientific).

Natural transformation assay. Stressors were tested either individually or combined to determine their effect on natural transformation. The stressors CMZ, FA, DBAC, FA and CHCl₃ were individually tested in different concentrations to determine the dosage effect per stressor. Specifically, the tested concentrations are CMZ (0.1, 0.15, 0.23, 0.28 and 0.32 μM), DBAC (1, 5, 10 and 20 μM), FA (100, 200, 400, 500, 600 and 800 μM) and CHCl₃ (0.2, 0.8, 1.6, 2 and 2.8 mM). Solar irradiation and its effect on natural transformation were obtained from an earlier study²⁷. With the exception of CMZ (which was detected in similar concentration ranges in wastewater³⁷) and solar irradiation (which represents the fluence detected in the KAUST, Saudi Arabia⁵⁰), the concentrations of chemicals used in this study were higher than that of environmental concentrations. Higher concentrations of chemicals were used due to the detection sensitivity limits of natural transformation frequencies (10⁻⁸ to 10⁻⁹) by the *A. baylyi* ADP1 system and the need to demonstrate a range that can reflect a potential concentration-dependent response. The potential cytotoxic impact of applied concentrations was verified on the basis of the comparison of the total cells number of treated and non-treated cells (Supplementary Figs. 1–3). Based on the obtained results from single-factor transformation experiments, one concentration per chemical stressor was used for combination experiments (Supplementary Fig. 8), specifically CMZ (0.28 μM), DBAC (5 μM), FA (500 μM) and CHCl₃ (1.6 mM). For the combination experiments, 41.8 mJ cm⁻² of solar irradiance was chosen in this study because, on the basis of our in-house tests, solar irradiation is a strong inducer of natural transformation compared with other chemical stressors and an overly high fluence of solar irradiation would mask the apparent effect on natural transformation by other chemical stressors. These chosen concentrations and fluence doses in combined permutations were also assessed to ensure no lethal effect on the growth of *A. baylyi* ADP1 (Supplementary Figs. 1–3). Transformation assay was carried out as described in refs. 14,15. Briefly, *A. baylyi* ADP1 cells were cultured overnight in liquid Luria-Bertani (LB) media supplemented with 50 μg ml⁻¹ rifampicin and 50 μg ml⁻¹ kanamycin. After 18 h of incubation at 37 °C, cells were diluted to an OD_{600nm} of 0.05 (approximating 2 × 10⁷ colony-forming units (CFU) ml⁻¹) for use in transformation experiments. Two micrograms of donor DNA was applied to each reaction with the required amount of stressor in antibiotic-free LB media. Reactions were incubated at 37 °C at 200 r.p.m. for 24 h. Following incubation, a series of dilutions were applied for each reaction in PPB (10⁻¹, 10⁻², 10⁻³, 10⁻⁴, 10⁻⁵ and 10⁻⁶). Then, 100 μl of 10⁻¹ diluted cells were plated in an LB media agar plate supplemented with 50 μg ml⁻¹ rifampicin, 50 μg ml⁻¹ kanamycin and 100 μg ml⁻¹ spectinomycin to determine the number of transformant cells. Additionally, 100 μl of 10⁻⁶ diluted cells were plated in an LB media agar plate supplemented with 50 μg ml⁻¹ rifampicin and 50 μg ml⁻¹ kanamycin to verify the total population of cells. Plates were incubated at 37 °C for 24 h for cells to grow. Afterwards, colonies were counted, and the frequency of transformation and transformation for each reaction was determined. The transformation frequency during 24 h of incubation was also analysed at 1, 3, 5, 7, 8 and 24 h to determine the starting point of transformation and the stability of transformed cells during 24 h of incubation (Supplementary Fig. 9).

Calculations. To determine the transformation frequency per reaction/technical replicate, the number of transformed cells was divided by the average of the total population as indicated in equation (1). Further, the transformation frequency of treated cells was normalized against the average of transformation frequency of control cells (equation (2)) to determine the transformation fold change.

$$\text{Transformation frequency} = \frac{\text{Number of transformed cells}}{\text{Average of total population}} \quad (1)$$

$$\begin{aligned} \text{Fold change for transformation} \\ = \frac{\text{Transformation frequency of treated cells}}{\text{Average of transformation frequency of control cells}} \end{aligned} \quad (2)$$

To calculate the theoretical range of combined stressors, the average of the transformation fold change per experiment for the single factor was applied into the Bliss equation⁵¹ as indicated in equation (3).

$\sum_i (1 - f_i(d_i)) = 1 - E$, which then simplifies to equation (3) when there are only two combined stressors.

$$1 - E = N - (R_{N=1} + R_{N=2}) \quad (3)$$

where $N = 2$, which is the number of combined stressors used in this study, R is the response per stressor and E is the theoretical expected outcome. Detailed derivatization of equation (3) from the Bliss equation is listed in Supplementary Method 1.1.

The theoretically expected outcome was compared against the outcome obtained via wet-bench experiments to denote the suitability of the Bliss equation (Fig. 4). In our study, the outcome of a single factor can fall within three possible results: (1) an increase in transformation frequency compared with the control, (2) no observed impact in transformation frequency compared with the control and (3) a decrease in transformation frequency when compared with the control. Since single stressors can result in a positive or a negative impact, when defining the type of interaction between such combined stressor pairings, outcomes were classified into four categories based on statistical testing of the combined outcome against outcomes obtained from individual stressors. The four categories are (1) additive, if the mean of the observed response is not statistically different from the mean of the calculated geometric addition derived from equation (3), (2) synergistic, if the mean of the observed response results in a significant increase in transformation fold change compared with both stressors and is significantly higher than the theoretical geometric addition (equation (3)), (3) neutral, if the mean of the observed response results in an insignificant difference compared with the transformation of the single stronger stressor (defined as the stressor that is inducing the comparatively higher transformation outcome of the pair) and (4) antagonistic, if the mean of the observed response falls below significantly compared with the single stronger stressor.

Mutagenicity test for tested stressors. Stressors evaluated in this study can affect natural transformation frequency by mutagenic-induced DNA damage. This can in turn trigger homologous recombination in a *recA*-dependent manner (that is, integration step of natural transformation), which was previously shown to be a molecular link between DNA repair and natural transformation^{14,15}. Mutagenicity assay was carried out as described by refs. 14,15,26. Briefly, TA100 cells were incubated overnight at 37 °C for 16 h in LB media supplemented with 25 μg ml⁻¹ of ampicillin. Cells were washed twice with PPB and measured at 660 nm to adjust the cell number to 2 × 10¹⁰ CFU ml⁻¹. After cell adjustment with PPB, 100 μl of cells were added in each reaction with the respective stressor to test. A positive control of 100 μg of sodium azide and a negative control with only PPB were included in all experiments. Samples of each concentration were done in three technical replicates and plated onto minimal glucose plates supplemented with

Vogel–Bonners salt solution⁵². After 48 h of incubation at 37 °C, the number of histidine revertant colonies was counted and normalized against the number of colonies derived from the negative control. Moreover, the standardized cells were grown in a separate plate along with a sterile disk that absorbed crystal violet to ensure that the cells contain *rfa* mutation, which allows higher permeability for larger molecules⁵³.

Measurements of cell membrane permeability. Stressors evaluated in this study can also affect natural transformation frequency by changing cell membrane permeability (that is, translocation step of natural transformation). To determine the effect on cell permeability, bacterial cells were stained with the BacLight Bacterial Viability Kit (Invitrogen by Thermo Fisher Scientific) with a final concentration of 30 µM. Briefly, *A. baylyi* was propagated and adjusted to cell density similar to the transformation assay setup where approximately 2×10^7 CFU ml⁻¹ was used per reaction. Each reaction was incubated for 2 h at 37 °C at 200 r.p.m. in an LB medium. The 2 h incubation was used as this coincides with the initial phase of natural transformation (Supplementary Fig. 9) and hence represents the phase where cell membrane permeability is induced to allow the first step of natural transformation (that is, translocation) to occur. Cells were then centrifuged at 13,000g for 30 s, diluted in 500 µl PPB, and stained with PI as described by the manufacturer. BD FACS LSRFortessa was used for the detection of PI fluorescence, with at least 1,000,000 events counted per sample. Gating for total cells was first performed on the basis of the forward scatter (FSC, size) versus the side scatter (internal complexity) plot (Supplementary Fig. 10a). The gating of total cells was further verified using SYTO9 staining for all cells as indicated in (Supplementary Fig. 10b). Briefly, gating for stained cells was accomplished using fluorescence-A (A: integral of the height and the width of the pulse area, which is typically used to measure and report fluorescence plot) versus forward scatter area (FSC-A). The FSC versus fluorescence-A plot was then used to gate singlet cells on the basis of their fluorescence intensity using PI-A versus FSC-A. The portion of cells gated in the PI (+) region corresponds to the frequency of stained cells due to cell permeability (Supplementary Fig. 10c). The frequency of PI (+) cells to total cells was determined, and the fold change in cell membrane permeability induced by the stressor was further calculated by normalizing the frequency derived from each treatment against the control. Gating regions are exemplified in Supplementary Fig. 10. All experiments were done in at least three biological replicates.

ROS production measurement assay. A single colony of *A. baylyi* ADP1 was cultured in LB Lenox medium at 37 °C, 200 r.p.m. to reach an exponential growth phase. The culture was washed once with phosphate-buffered saline (PBS) buffer and a cell suspension was made with the cell number 2×10^7 CFU ml⁻¹ in PPB, similar to the transformation assay. Then cells were stained with 5 µM of CM-H2DCFDA (Invitrogen, Thermo Fisher Scientific) at 37 °C for 30 min. Since cells had to be stained before treatment, cells were incubated in PPB instead of LB to prevent further microbial growth and avoid clogging the flow cytometer's injector. Then 100 µl of the stained cells were mixed with 100 µl of PBS buffer that has either no stressor, CMZ (0.28 µM), DBAC (5 µM), FA (500 µM) or CHCl₃ (1.6 mM). A sample lacking fluorescent dye was also included as a control for autofluorescence. Cells were treated with 10 µM, 100 µM and 1 mM hydrogen peroxide for positive control. The reactions were incubated in the dark at 37 °C and samples were collected at 30 min timepoint. Collected samples were placed immediately on ice. Subsequently, each sample was diluted tenfold in 1× PBS buffer and determined for ROS production by BD LSRFortessa Cell Analyzer (Becton Dickinson). A total of 50,000 events were analysed at a speed of 2 µl s⁻¹. Fold change analysis was done in a similar manner as described previously for cell membrane permeability.

Gene expression determination. Since different factors can affect natural transformation by triggering different pathways in the cell,

the expression levels of selected genes that have been shown before to contribute to natural transformation by different stressors^{14,15} were analysed by quantitative reverse transcription PCR. Genes that were selected for gene expression profiling are *comA* and *pilX* as DNA translocation genes and *recA*, *ddrR* and *uvrB* as DNA integration genes. More details on the quantitative reverse transcription PCR experiments are provided in Supplementary Information (Supplementary Method 1.2 and Supplementary Table 7).

Effect of FA and CHCl₃ on donor DNA. Both FA and CHCl₃ have been previously shown to have an impact on the cell membrane and DNA^{29,30}. To determine whether the detrimental effect of FA and CHCl₃ on transformation is due to direct interaction with the donor DNA, a transformation experiment was carried out using purified donor DNA that has been separately treated with either FA or CHCl₃ first, before introducing it to *A. baylyi* ADP1. Further experimental details are provided in Supplementary Method 1.3.

Collection of chlorinated effluent and sample analyses. Chlorinated effluent were grab collected during the period of 5 October to 7 November 2021. Samples were reconstituted via solid-phase extraction (SPE) for characterization by liquid chromatography–tandem mass spectrometry. All details on sample collection, SPE and chemical characterization can be found in Supplementary Method 1.4.

Effect of chlorinated effluent on natural transformation. To test the effect of chlorinated effluent on natural transformation, *A. baylyi* was propagated and prepared in a similar manner as described earlier in natural transformation experiments. Lyophilized chlorinated effluent samples were diluted in 500 µl biological grade water. Each sample was tested in three biological replicates. In each experiment, 100 µl of the SPE sample (reconstituted in 2,000×) was added to the reaction tube. As for control, Milli-Q water of equal volume to that of the SPE sample was added to the reaction.

Mitigating natural transformation through eDNA concentration. To minimize the impact of existing stressors in treated water and their subsequent impact on natural transformation, we investigated the impact of eDNA concentration to competent cell ratio. Transformation assays were applied using different concentrations of donor DNA supplemented in different reactions. The tested concentrations included 0.05, 0.1, 1, 2 and 4 µg ml⁻¹. All transformation reactions were exposed to 41.7 mJ cm⁻² solar irradiance, and 5 µM of DBAC as this paired combination resulted in an additive interaction where natural transformation frequency can reach up to 2.8-fold higher than that of control.

Statistical analyses. All experiments were conducted independently in at least biological triplicates. All data were expressed as mean ± standard deviation (s.d.). Significant differences between different treated/control groups were analysed using a one-way analysis of variance (ANOVA) with the Holm–Bonferroni method, or by the *t*-test method when indicated. *P* values less than 0.05 were considered to be statistically significant.

Data availability

All data generated or analysed during this study are included in this published article and its Supplementary Information files. The full dataset of processed data will be available upon request.

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Author contributions

B.A. designed the overall experiments for this study and performed all analyses. B.A. performed the individual and combined transformation assays for four out of five stressors. B.A. also carried out mutagenicity assays for three out of five studied stressors, ran all quantitative PCR experiments and cell permeability assays, contributed to ROS

production assay and carried out transformation assays with treated water and at different donor DNA concentrations. B.A. wrote the full manuscript and illustrated all provided figures. D.M.-C. contributed to the initial planning for this study, performed mutagenicity assay for two out of five studied factors, and contributed to revising the manuscript. T.W. performed transformation assay for individual and combined experiments for CHCl₃. T.W. also optimized the ROS production assay and performed the required experiments. M.A.G. provided the prediction model in the initial version of the manuscript. F.B. collected chlorinated effluent samples, performed the SPE analysis for collected samples and contributed to the characterization of chemicals in chlorinated effluent samples. D.D. provided the *A. baylyi* ADP1 reporter system and provided feedback on the transformation experiments. T.-M.L.-K. supervised the establishment of the prediction model in the initial version of the manuscript. P.-Y.H. supervised and managed the project, and contributed to the writing and revising of the manuscript. All authors provided feedback and discussed the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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