
Combined effects of light pollution and the insecticide malathion on the freshwater crustacean *Daphnia magna*

Defêche Héloïse

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de Master en Biologie des Organismes et Ecologie

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Preface

This Master's thesis was written in the form of a scientific article in order to train in this type of scientific writing, which is relevant for my future academic and professional career.

Artificial intelligence tools (ChatGPT 5.2) were used occasionally to improve scientific English and to assist with minor modifications in R coding. All analyses, interpretations, and conclusions remain the author's own.

Acknowledgement

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Abstract

Freshwater ecosystems are increasingly exposed to multiple anthropogenic stressors, including chemical pollution and artificial light at night (ALAN). Although their individual effects on aquatic organisms are well documented, their combined impacts remain poorly understood. In this study, we investigated the individual and combined effects of the organophosphorus insecticide malathion and ALAN on the freshwater cladoceran *Daphnia magna*. An acute toxicity test (48 h) and a sub-chronic exposure (15 days) were conducted under natural light–dark cycles or with artificial light during the night. Endpoints included immobilization, morphology, life-history traits, and swimming behaviour. Malathion toxicity was reduced under light exposure in the acute test, likely reflecting changes in pollutant availability. In the sub-chronic experiment, ALAN affected growth, eye morphology, and reproduction, whereas malathion showed limited effects at the tested concentration. These results highlight the importance of considering multiple stressors when assessing ecological risks in freshwater environments.

1. Introduction

The rapid growth of the human global population, coupled with industrialization and urbanization, has introduced numerous challenges to ecosystems. Among these is the significant increase in pollution, which can manifest in various forms, including air, noise, light, water, and soil pollution (Jindal, Kumar, and Kumar 2020). These forms of pollution have permeated all types of environmental matrices and ecosystems, including the atmosphere as well as a broad range of terrestrial, freshwater, estuarine, and marine environments (Hill 2020). Aquatic environments are particularly vulnerable to pollution notably due to humanity's long-standing tendency to settle near water. Today, over half the global population resides within 3 kilometers of an inland water body (Kummu et al. 2011), placing freshwater ecosystems under increasing anthropogenic pressure, notably from light and chemical pollution.

The presence of artificial light at night (ALAN) has grown substantially over recent decades, with satellite-detected light having increased by 49% between 1992 and 2017 (Sánchez de Miguel et al. 2021). Given the exceptional biodiversity and ecological importance of freshwater ecosystems, this rapid expansion of nocturnal illumination raises particular concern. Light pollution is known to affect multiple aspects of organismal biology, including behaviour, physiology, and its increasing prevalence therefore has the potential to disrupt freshwater ecosystem structure and functioning, with consequences that may propagate across trophic levels (Ahmed et al. 2022; Desforges et al. 2022).

Light plays a fundamental role in the lives of most organisms, acting both as a driver of photosynthesis or as a source of information influencing behaviour and physiology. Over evolutionary time, life has adapted to the natural light-dark cycles of the Earth, and these fluctuations have become essential cues for regulating a wide range of biological processes (Bhadra et al. 2017). ALAN poses two primary problems. First, it disrupts the natural day-night (light-dark) cycle and reaches environments that were historically untouched by artificial illumination. Second, the spectral composition of ALAN differs significantly from natural nocturnal light sources such as sunlight, moonlight, or starlight (Gaston et al. 2015). Since light is one of the primary environmental cues regulating biological rhythms, light pollution has the potential to disrupt the behaviour and physiology of organisms across multiple trophic levels (Thoré et al. 2024).

Animal behaviour is often the first aspect to change under artificial light, and these behavioural changes are frequently maladaptive, potentially reducing the organism's overall fitness (Ganguly and Candolin 2023). ALAN can alter foraging behaviour, with nocturnal species

reducing their foraging activity due to increased illumination, while diurnal species may extend their foraging into the night (Davies and Smyth 2018; MacGregor et al. 2015). This shift can increase predation pressure on species that are not normally exposed to such risk during nighttime, potentially destabilizing predator-prey dynamics (Davies and Smyth 2018). Additionally, changes in light exposure can interfere with reproductive behaviour by disrupting hormone production and circadian timing. For example, ALAN has been shown to disturb the synchronized mass spawning of corals, a process tightly linked to natural light cues, mainly moonlight (Ganguly and Candolin 2023; Davies et al. 2023). Migration is another key behaviour impacted by light pollution. Many organisms are either attracted or repelled by light which can interfere with their natural migratory routes (Burt et al. 2023; Korpach et al. 2022).

Aquatic ecosystems are rarely exposed to stressors in isolation, rather, they are subjected to multiple stressors. While the effects on individual stressors have been extensively studied across various biological levels, the interactions between them remain far less understood (Birk et al. 2020). Notably, the combined impact of multiple stressors often cannot be predicted by simply summing their individual effects (Pirotta et al. 2022). As illustrated in Annex 1, stressor interactions can take several forms. These interactions may be additive, where the combined effect equals the sum of each stressors. Alternatively, they may be multiplicative, which is frequently the case with mortality effects; once an individual has died due to one stressor, it cannot be affected again by another. In some cases, the combined effect is equal only to that of the more impactful stressor, a scenario referred to as dominance (Côté et al. 2016). More complex interactions can occur when the joint effect of two stressors is greater than their sum, known as a synergistic effect. Conversely, if the combined impact is less than the additive effect, it is described as an antagonistic interaction (Côté et al. 2016). In some cases, stressors may even have opposing effects, potentially masking or reversing one another's impacts (Annex 2) (Côté et al. 2016).

Among the major chemical stressors affecting freshwater ecosystems are synthetic chemicals released through industrial and agricultural activities. Approximately 300 billion kilograms of synthetic chemicals are believed to enter freshwater ecosystems each year as a result of diverse industrial activities (Yang et al. 2021). Among these, insecticides are particularly prevalent. Global pesticide use is estimated at approximately 2 million tonnes per year, with insecticides accounting for 29.5% of that amount (Sharma et al. 2019).

These insecticides encompass a wide range of chemical families with different modes of action, including organophosphorus insecticides. Their main action mode is the phosphorylation of acetylcholine esterase which results in its inhibition (Chambers et al. 2010). Inactivation of this

enzyme prevents the degradation of acetylcholine, causing its accumulation within cholinergic synapses and neuromuscular or glandular junctions, which in turn results in excessive cholinergic activity (Chambers et al. 2010).

One widely used organophosphorus insecticide, and the focus of the present study, is malathion (diethyl 2-dimethoxyphosphinothioylsulfanylbutedioate or $C_{10}H_{19}O_6PS_2$) (Deka and Mahanta 2016). Malathion (Annex 3) is applied across a broad range of sectors, from animal husbandry, where it is used to control ectoparasites, to agriculture, where it protects field crops from insect pests. It is also employed in human health to treat head lice and to control disease vectors, including mosquitoes (Toumi et al. 2015; Jensen and Whatling 2010). The extent of malathion use varies geographically. In the United States, it remains one of the most widely applied insecticides, with annual releases estimated between 10 and 14 million kilograms (Iii and Relyea 2015). Although malathion has been restricted in the European Union since 2008, it continues to be detected in surface waters, where concentrations frequently exceed national environmental quality threshold (Arnal et al. 2025). Due to regional differences in malathion usage and application practices, its environmental concentrations can vary widely. For example, malathion concentrations measured in surface waters in Mexico range from 771.49 to 863.49 $\mu\text{g L}^{-1}$, whereas much lower concentrations have been reported in Spain, ranging from 3.01 ng L^{-1} to 320.35 ng L^{-1} (Vasseghian et al. 2022; Masiá et al. 2015; Silva-Madera et al. 2021)

Malathion toxicity results from its bioactivation via cytochrome P450-mediated oxidative desulfuration, which converts the parent compound into its more toxic metabolite, malaoxon (Annex 3). Malaoxon is responsible for most acetylcholinesterase inhibition associated with malathion exposure (Reed and Rubin 2014). Detoxification pathways involving carboxylesterases can partially mitigate toxicity by hydrolysing malathion and malaoxon into less toxic metabolites such as malathion or malaoxon monocarboxylic acid (MCA) and dicarboxylic acid (DCA) (Reed and Rubin 2014; Barata et al. 2004). Because these enzymatic pathways are present not only in target species but also in many non-target organisms, malathion poses a risk to a wide range of taxa including aquatic invertebrates (Newhart 2006).

Among non-target aquatic invertebrates, cladocerans of the genus *Daphnia* are widely used as bioindicator species in freshwater ecosystems due to their high sensitivity to environmental stressors and their central ecological role as primary consumers of phytoplankton and as a key food source for higher trophic levels (Miner et al. 2012; Leung et al. 2025). Disruption of *Daphnia* can therefore have cascading effect on freshwater food webs. Numerous studies have demonstrated that *Daphnia* species are sensitive to malathion under both acute and chronic exposure on their survival development and reproduction (Toumi et al. 2015; Trac et al. 2015).

In addition, *Daphnia* is highly responsive to light conditions, as light cues regulate behaviours such as diel vertical migration. ALAN has been shown to affect their physiology, life-history traits and behaviour (Li et al. 2022; Ganguly and Candolin 2023)

In this context, *Daphnia magna* is an excellent model organism for investigating the combined effects of ALAN and malathion exposure. Both ALAN and malathion are known to affect similar biological traits, including behaviour, growth, and reproduction, suggesting that their combined exposure may result in interactive effects. Moreover, light pollution may indirectly influence malathion toxicity by altering its environmental fate and degradation (Bavcon Kralj et al. 2007), making *D. magna* particularly suitable for studying both direct and indirect stressor interactions.

Beyond its ecological relevance, *D.magna* is also widely used in ecotoxicological research due to its short life span, rapid reproduction, and short generation time, which allow the assessment of life-history responses over relatively short experimental periods. It is easy to culture and maintain under laboratory conditions and can reproduce asexually under favourable conditions, producing genetically identical clones that reduce genetic variability and enhance experimental reproducibility (Reilly et al. 2023; Ahmed 2023). Together, these characteristics make *D. magna* a powerful model for disentangling the effects of multiple anthropogenic stressors in freshwater ecosystems.

Here, we investigated the individual and combined effects of artificial light at night and the organophosphorus insecticide malathion on *Daphnia magna* under acute and sub-chronic exposure scenarios. Specifically, we assessed the effects of each stressor alone, as well as whether ALAN modifies malathion toxicity, leading to non-additive responses in survival, behaviour, and life-history traits. This approach improves our understanding of how multiple anthropogenic stressors jointly affect freshwater organisms.

2. Materials and methods

2.1 *Daphnia magna* breeding and maintenance

Clonal lineages of *Daphnia magna* were originally established at the Freshwater Ecology, Evolution & Conservation laboratory of KU Leuven (Leuven, Belgium) from dormant (resting) eggs collected in a shallow eutrophic pond in Flanders, Belgium (Langerodevijver; 50°49'42.2" N, 4°38'23.7" E). Sediment from the upper 2 cm layer of the pond was sampled between April and May 2018 and spread as a thin layer on white trays filled with dechlorinated tap water. Trays were incubated at 20 ± 1 °C under a 16 h light : 8 h dark photoperiod and checked every two days for hatchlings.

Because resting eggs in Daphniidae are produced through sexual reproduction and are therefore genetically unique, each newly hatched individual was used to establish a distinct clonal lineage. From these hatchlings, 20 unique clones per species were randomly isolated and maintained separately. For the present study, three clonal lineages (DM7, DM9, and DM22) were obtained from KU Leuven in early 2025. Upon arrival, clones were cultured under standard laboratory conditions and maintained for at least three asexual generations prior to the start of the experiments to minimize potential maternal or transport-related effects.

Cultures were maintained in a controlled environmental room at 19 °C under a 16 h light : 8 h dark photoperiod (details in Annex 4) in 500 mL glass jars containing ADaM (Aachener Daphnien Medium) medium (see composition in Annex 5) (Klüttgen et al. 1994). The culture medium was renewed three times per week (On Monday, Wednesday and Friday) by replacing approximately three-quarters of the old medium. The new neonates were removed from the jars while the adults were kept. Neonates from the second brood were used to create new generation while the rest were killed using Javel. On water changes days, individuals were fed with 500 µL of the green alga *Scenedesmus obliquus* (7.7×10^6 cells mL⁻¹ per jar) (Annex 6).

2.2 Experimental Setup

The study included two experiments: an acute exposure (48 h) and a sub-chronic exposure (15 days). Neonates less than 24 h old were used for all experiments to ensure uniformity in developmental stage and to maximize sensitivity to environmental stressors.

Two incubators were used to simulate two light conditions. The control incubator reproduced natural light conditions, in which *Daphnia magna* were exposed to approximately 1200 lux provided by a ClassicLED G2 LED light (spectral composition shown in Annex 7) for 16 h per day, mimicking a natural day–night cycle. The second incubator simulated light pollution conditions. In this setup, the same 16 h daytime illumination was supplemented with a low-

intensity light source from the same lamp model (Annex 7) that remained on throughout the night, maintaining a constant illumination of 20 lux.

2.3 Acute toxicity

The acute toxicity (see Annex 8.1) test was conducted using a single clonal lineage (DM9) to minimize genetic variability. Neonates (< 24 h old) from the second brood were exposed to a range of malathion concentrations (0; 0,9; 1,15; 1,45; 1,8; 2,25; 2,8; and 3,5 µg/L). Preliminary tests were conducted using concentration values reported in the literature (Toumi et al. 2015) to identify an appropriate concentration range for the dose–response analysis.

Daphnia were placed in glass test tubes (25 ml capacity) containing 10 ml of test medium. Prepared from ADaM water and the appropriate malathion concentration. Malathion solutions were prepared from a freshly made stock solution (10 µg/L) on the morning of exposure. Five neonates were randomly placed in each tube, and tubes were randomly assigned to light treatments and concentration. Four replicates were used for each exposure condition and concentration. During the 48h exposure period, the organisms were not fed, and the medium was not renewed, following OECD 202 guidelines.

The number of immobilized *Daphnia magna* individuals was recorded after 24 and 48 hours of exposure. Following OECD 202 guidelines, individuals were classified as immobilized if they showed no movement for 15 seconds after gentle stimulation.

2.4 Sub-chronic toxicity

For the sub-chronic test (see Annex 8.2 and 8.3), three clonal lineage (DM7, DM9 and DM22) were used. Ten neonates per clone (< 24 h old) were individually placed in glass vessels containing 50 ml of either medium or medium supplemented with malathion (0.2 µg.L⁻¹). The vessels were randomly assigned to light treatments. This concentration was selected based on literature values (Toumi et al. 2015) and preliminary tests, as it induced sub-lethal effects without causing mortality and falls within the range of environmentally relevant concentrations.

Several endpoints were evaluated, including age at first brood, number of neonates per adult, average swimming distance and body and eye size measured at day 0, 7, and 14.

Size measurements were obtained from images captured using a digital microscope. Images were acquired with NIS-Elements software and subsequently analysed using ImageJ. Body size

was measured as the distance from the top of the head to the base of the spine, and eye diameter was measured at its widest point.

Behavioural assays were conducted on day 15 using video recordings acquired with a DanioVision observation chamber and analysed using EthoVision XT 14 software. Individuals were placed individually in six-well plates, with one *Daphnia magna* per well containing 9 mL of their respective experimental medium. Prior to recording, individuals were acclimated to the observation chamber for 5 min under 100% standardized light conditions. All behavioural assays were conducted between 10:00 and 13:00 to limit circadian variation and lasted 10 min per individual.

Throughout the experiment, organisms were fed every two days following water renewal. Water renewal was performed using the same procedure as for stock cultures. During each feeding event, *Daphnia magna* were supplied with 200 μ L of algal suspension per jar. The ADaM medium was prepared prior to the start of the experiment and stored at 19°C. A malathion stock solution (10 μ g L⁻¹) was prepared in 100% ethanol and stored at -20 °C. Fresh working solutions were prepared at each water renewal.

2.5 Data analysis

All analyses were performed using R (4.4.2) in RStudio. A significance threshold of $\alpha = 0.05$ was applied for all statistical tests. Data were compiled in Microsoft Excel and imported into R for analysis and visualization (see table of used packages in Annex 9).

For the acute test, dose-response relationships were analysed using the R package “drc” to estimate EC50 values when model convergence allowed reliable estimation. When the EC50 couldn't be determined, the effects of pollutant concentration, ALAN, and their interaction on immobilization were analysed using binomial generalized linear models.

For the sub-chronic experiment, body size was analysed using linear mixed-effects models (LMM) including light treatment and sampling day, malathion exposure, and the interaction between light treatment and sampling day as fixed effects. Individual identity was included as a random intercept to account for repeated measurements over time. Eye diameter at day 7 and day 14 was analysed using LMM. Eye diameter was the response variable, body size at the corresponding day was included as a covariate, and ALAN and malathion exposure were included as fixed effects. Clone identity was included as a random intercept. Model assumptions were checked graphically, and fixed effects were tested using Type III Wald chi-square tests.

Age at first brood was analysed using survival analysis due to right-censoring of individuals that did not reproduce. Kaplan–Meier estimators were used for descriptive purposes, followed

by Cox proportional hazards models (Austin 2017). When the proportional hazards assumption was violated, time-dependent covariates were included. The probability of producing at least one brood was analysed using a binomial generalized linear mixed model, with ALAN, malathion exposure, and their interaction included as fixed effects and clone identity included as a random intercept. Total fecundity was analysed using a zero-inflated negative binomial generalized linear mixed model to account for excess zeros. Model assumptions were assessed and verified using residual diagnostics and Q–Q plots

Body size at day 14 was included as a covariate, ALAN and malathion exposure as fixed effects, and clone identity as a random intercept. Interaction terms were tested but removed when unsupported. Model assumptions were checked graphically, and fixed effects were assessed using Type II Wald chi-square tests.

3.Results

3.1. Acute test

At 48h under no-light conditions, a clear concentration-response relationship was observed. Using a two parameters log-logistic model with fixed limits (0-1), the estimates EC50 was 1,35µg/l ($p < 0.001$). As shown in Figure 1 the EC50 under light pollution conditions could not be estimated due to the absence of a response plateau, indicating that the maximum level of immobilization was not reached within the tested concentration.

To assess the effect of ALAN on immobilization at 48h, we fitted a binomial generalized linear model including concentration, light treatment, and their interaction. As expected, the model revealed a strong and highly significant positive effect of malathion concentration on immobilization ($\beta = 1.72$, $\chi^2 = 66.68$, $p < 0.001$), indicating that increasing malathion concentration markedly increased the probability of immobilization. In contrast, artificial light at night (ALAN) alone had no significant effect on immobilization ($\chi^2 = 1.32$, $p = 0.25$). However, the interaction between concentration and ALAN was significant ($\beta = -0.76$, $\chi^2 = 5.33$, $p = 0.02$), demonstrating that light pollution significantly reduced the strength of the concentration-response relationship.

We also tested this model on the proportion of organisms immobilized at 24 hours, this revealed that light pollution and its interaction with concentration had no significant effect on the immobilization, which depended only on the concentration ($\chi^2 = 0.39$, $p = 0.54$)

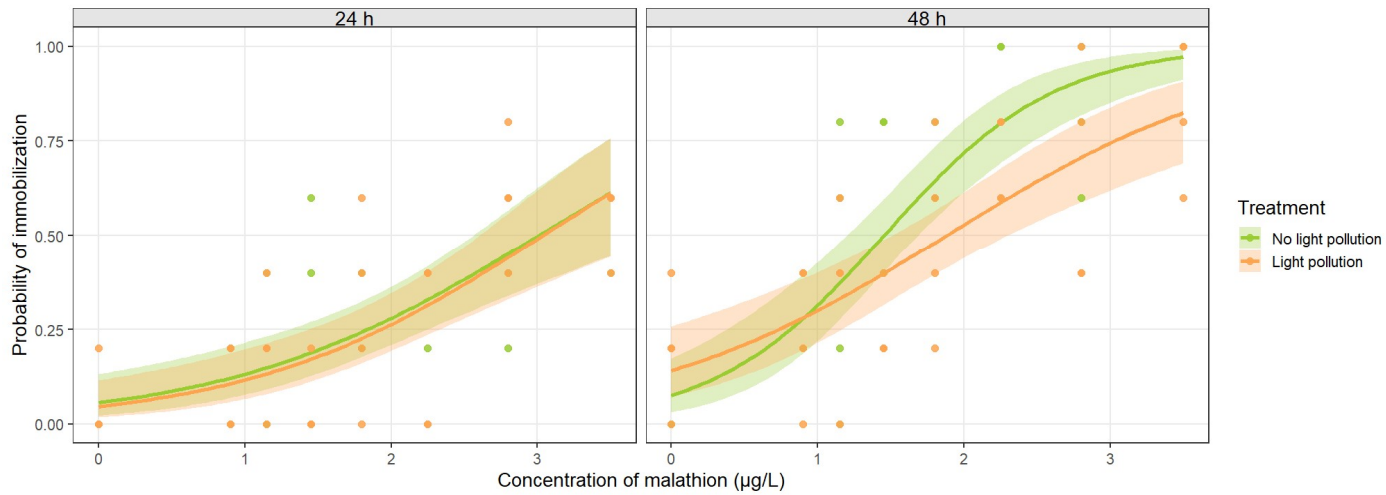


Figure 1. Concentration–response relationships for *Daphnia magna* immobilization after 24 h (left panel) and 48 h (right panel) of malathion exposure under no-light (control, green) and light pollution (orange) conditions. Points represent observed proportions, solid lines indicate predictions from binomial logistic models, and shaded areas represent 95% confidence intervals.

3.2. Sub-chronic experiment

3.2.1. Morphology

As expected, *D. magna* body size increased strongly with sampling day (day 7: $\beta = 1.44$, $p < 2 \times 10^{-16}$; day 14: $\beta = 2.67$, $p < 2 \times 10^{-16}$). Malathion exposure had no significant effect on body size ($\beta = -0.016$, $p = 0.68$), nor did ALAN show an overall main effect on body size across sampling days (ALAN; $\beta = -0.002$, $p = 0.97$).

However, ALAN significantly altered growth trajectories, as indicated by a significant ALAN $\times$ day interaction. As shown in Figure 2, individuals exposed to ALAN were significantly larger at day 7 than those under control conditions ($\Delta = +0.19$ mm, $p = 0.00018$), whereas no significant difference was detected at day 14 ($\Delta = +0.08$ mm, $p = 0.13$). Post hoc Tukey-adjusted comparisons based on estimated marginal means confirmed the absence of differences between light treatments at day 0 and day 14, but revealed a significant positive effect of ALAN at day 7.

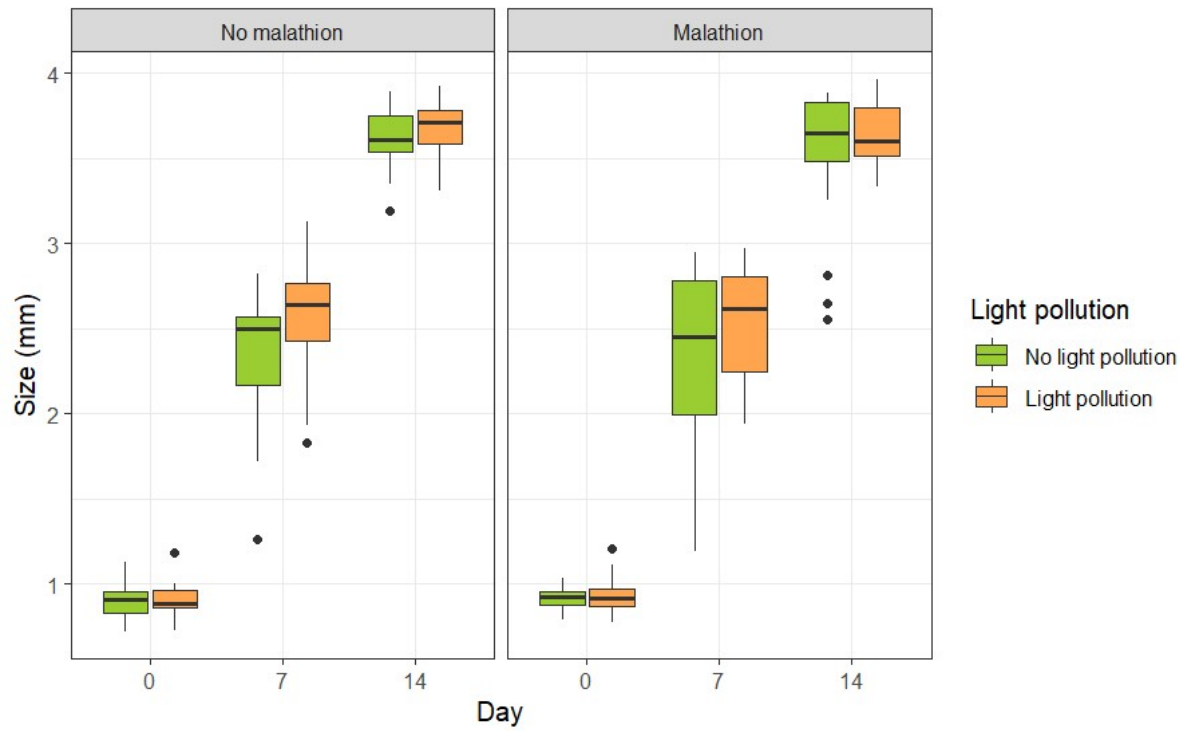


Figure 2. Boxplots showing the distribution of *Daphnia magna* body size (mm) at days 0, 7 and 14 under malathion exposure and control conditions, in the presence (orange) or absence (green) of artificial light at night (ALAN). Panels represent malathion exposure (left: no malathion; right: malathion). Boxplots display median values (horizontal lines), interquartile ranges (boxes), and minimum and maximum values excluding outliers (whiskers); points indicate outliers.

Eye diameter was strongly related to body size at both sampling times. At day 7 (Figure 3), body size showed a strong and highly significant effect on eye diameter ($\chi^2 = 148.09$, $p < 2.2 \times 10^{-16}$), whereas neither artificial light at night (ALAN; $\chi^2 = 1.61$, $p = 0.20$) nor malathion exposure ($\chi^2 = 0.55$, $p = 0.46$) had a significant effect. At day 14, eye diameter also increased significantly with body size ($\chi^2 = 10.90$, $p < 0.001$). Malathion exposure did not affect eye diameter at this stage ($\chi^2 = 0.0074$, $p = 0.93$), while ALAN exposure showed a small but significant effect, resulting in a slight increase in eye diameter ($\chi^2 = 5.25$, $p = 0.02$), as illustrated in Figure 4.

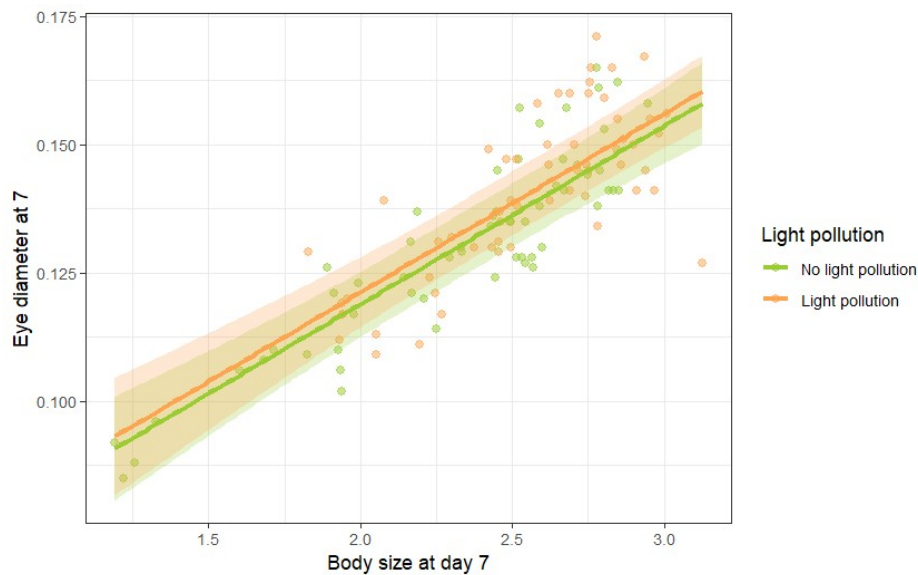


Figure 3. Eye diameter at day 7 as a function of body size at day 7 in *Daphnia magna*. Points represent individual measurements. Solid lines correspond to predictions from a linear mixed-effects model, and shaded areas indicate 95% confidence intervals. Colours denote light pollution treatments.

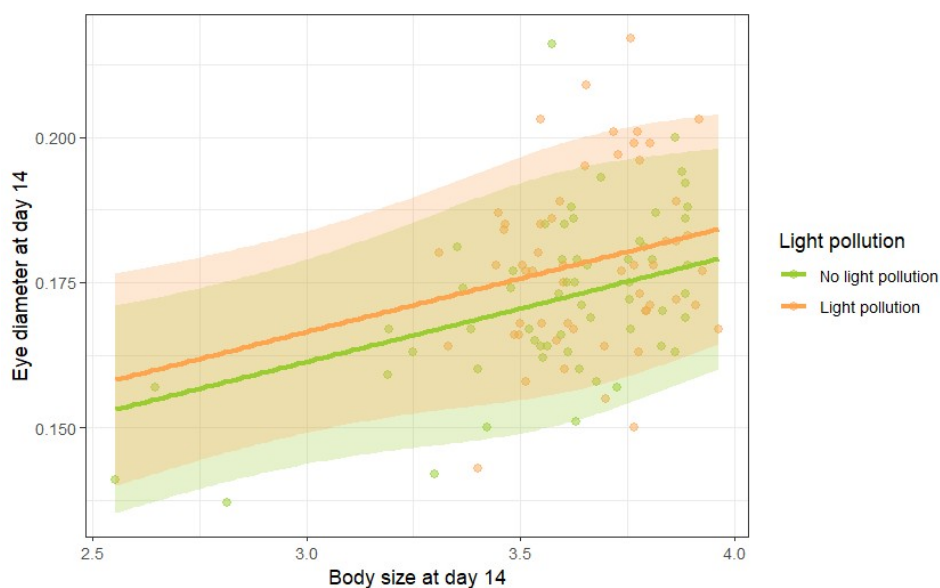


Figure 4. Eye diameter at day 14 as a function of body size at day 14 in *Daphnia magna*. Points represent individual measurements. Solid lines correspond to predictions from a linear mixed-effects model, and shaded areas indicate 95% confidence intervals. Colours denote light pollution treatments.

3.2.2. Population growth

Kaplan–Meier estimates showed that artificial light at night advanced the onset of reproduction across both malathion treatments, with the strongest differences observed around days 11–12 (Figure 5). Statistical analyses supported this pattern, indicating that ALAN significantly accelerated reproductive onset at day 11; however, this effect progressively weakened over time and became negligible after day 12. A log-rank test stratified by malathion treatment did not reveal a significant overall difference in time to first reproduction between light treatments ($\chi^2 = 2.9$, $p = 0.09$), reflecting the transient nature of the effect, which was restricted to early reproductive events.

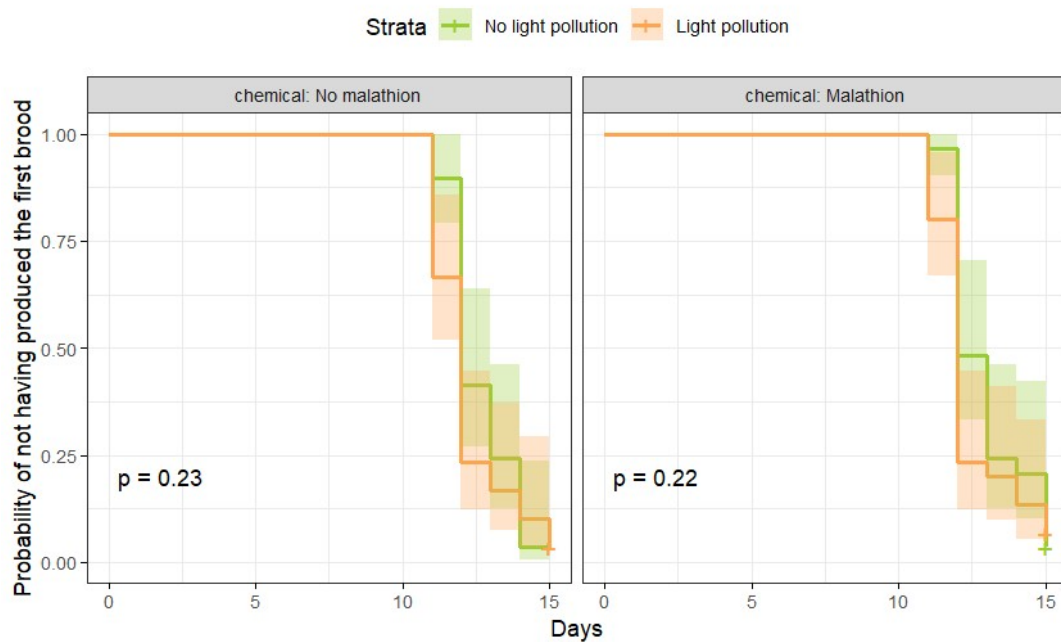


Figure 5. Kaplan–Meier curves showing the probability of not having produced the first brood over time in *Daphnia*. Curves are shown separately for malathion treatments (No malathion, Malathion) and coloured according to light pollution conditions (No light pollution, Light pollution). Shaded areas represent 95% confidence intervals. Individuals that did not reproduce during the experimental period were right-censored. P-values shown on Kaplan–Meier plots correspond to log-rank tests comparing survival curves across the entire experimental period.

To complement the analysis of reproductive timing, we used a binomial generalized linear mixed model including clone identity as a random effect to test whether the probability of producing at least one brood depended on experimental conditions. This analysis revealed no significant effect of light pollution (ALAN; $\chi^2 = 0.16$, $p = 0.69$), malathion exposure ($\chi^2 = 0.18$, $p = 0.67$), or their interaction ($\chi^2 = 0.17$, $p = 0.68$) on reproductive probability.

Total number of neonates (Figure 6) was analysed using a zero-inflated negative binomial generalized linear mixed model, with ALAN and malathion exposure as fixed effect and with clone as random effect. The results revealed that ALAN exposure increased by 30% the average total neonate produced ($\chi^2 = 6.00$, $p = 0.014$). In contrast malathion exposure revealed no

significant effect ($\chi^2 = 0.48$, $p = 0.49$) and the interaction between both was removed from the model since it was not supported.

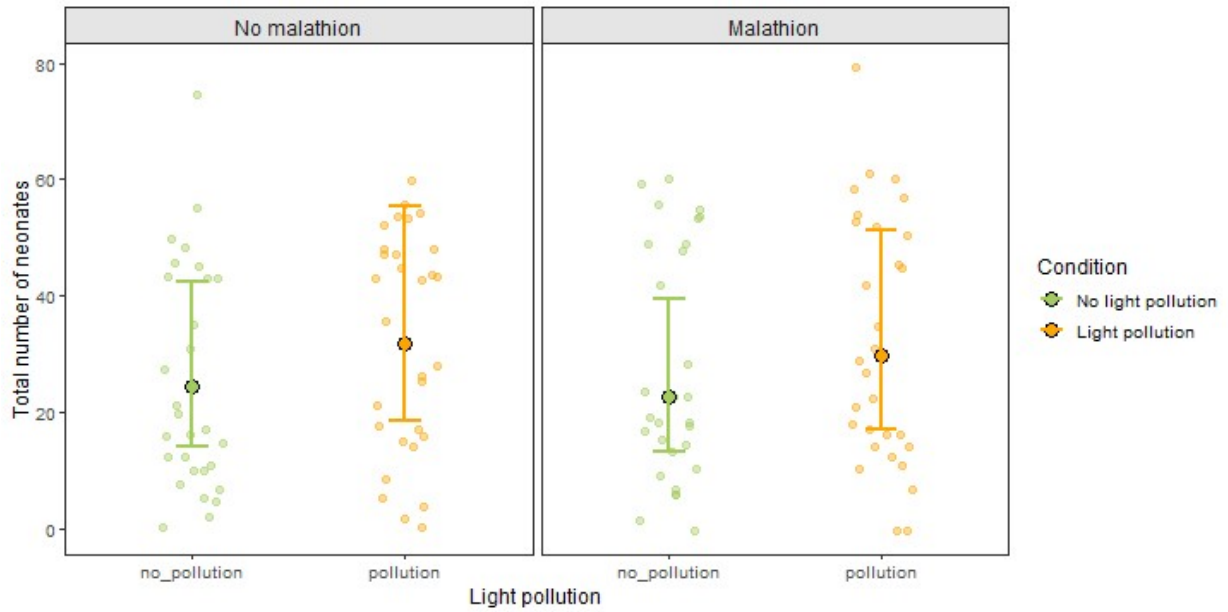


Figure 6. Total fecundity under light pollution and malathion exposure. Total number of neonates produced per individual under control (no malathion, left panel) and malathion (right panel) conditions. Points represent individual observations, with individuals exposed to light pollution shown in orange and control individuals (no light pollution) shown in green. Large symbols and vertical bars indicate model-predicted means and 95% confidence intervals obtained from a zero-inflated negative binomial generalized linear mixed model.

3.3. Behaviour

Neither body size ($\chi^2 = 0.84$, $p = 0.36$), light pollution ($\chi^2 = 1.52$, $p = 0.22$), nor malathion exposure ($\chi^2 = 1.58$, $p = 0.21$) had a significant effect on the mean swimming distance of *Daphnia magna*. Clone identity accounted for part of the variability but did not alter the absence of treatment effects (Figure 7). Nevertheless, clone identity explained part of the variability in swimming activity. While exploratory analyses suggested clone-specific patterns in the response to light pollution, these differences were not formally tested in the main model and may have contributed to the absence of an overall treatment effect.

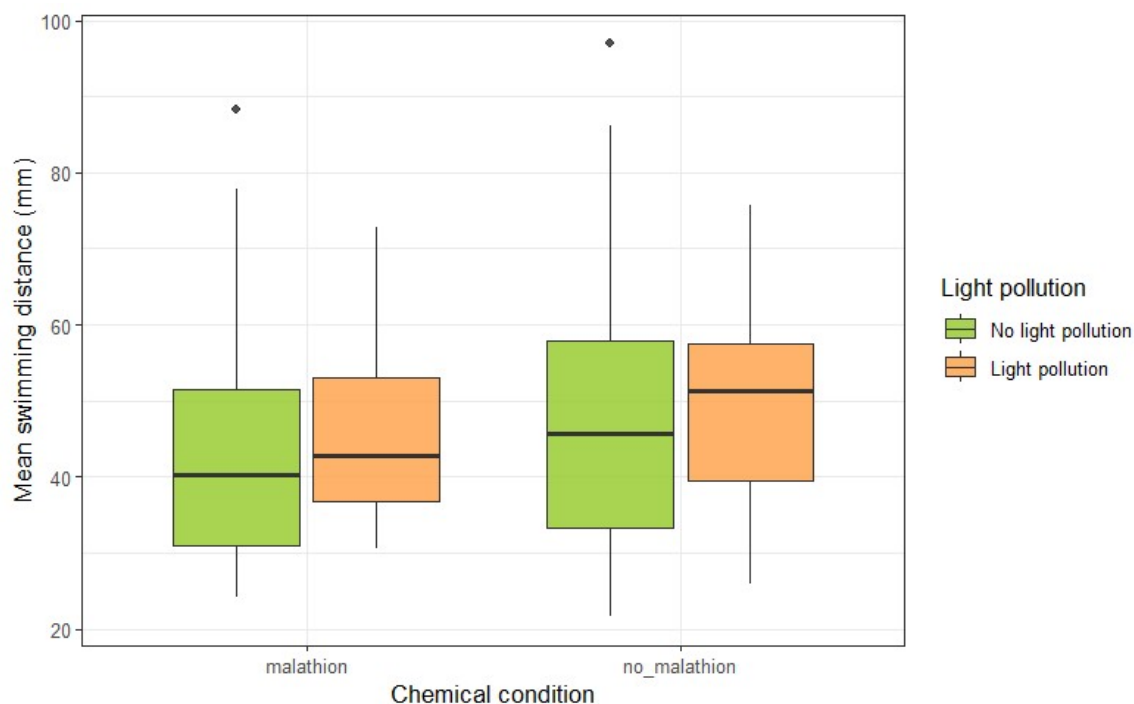


Figure 7. Mean swimming distance of *Daphnia magna* under malathion and control conditions, in the presence or absence of artificial light at night (ALAN). Boxplots show the distribution of mean swimming distance (mm) measured during the behavioural assay. Boxes represent interquartile ranges with median values indicated by horizontal lines; whiskers show the minimum and maximum values excluding outliers, which are displayed as individual points. Colours indicate light treatments (green: no light pollution; orange: light pollution).

4. Discussion

The present study investigated the combined effects of malathion exposure and ALAN on the survival, morphology, reproduction, and behaviour of *Daphnia magna* under both acute and sub-chronic experimental conditions.

The 48 h EC₅₀ estimated under normal light conditions (1.35 µg/L) was higher than values reported in the literature, indicating a lower sensitivity of *D. magna* to malathion in the present experiment. For instance, Toumi et al. reported 48h EC₅₀ values ranging between 0,36 and 0,46 µg/L for three *D.magna* strains (Toumi et al. 2015). An other study on *D.magna* has reported intermediate sensitivity, with EC₅₀ values around 0.90 µg/L, highlighting substantial variability among experiments (Ren et al. 2007).

Differences in sensitivity could potentially be attributed to genetic or environmental differences among clones, as local adaptation may influence stress responses (He et al. 2025). However, this explanation remains uncertain, as the strains used by Toumi et al. originated from geographically distinct locations (UK, Japan and France) yet exhibited relatively similar EC₅₀ values.

An alternative explanation lies in differences in malathion formulation. The formulation of malathion differed between studies: Toumi et al. (2015) used a commercial product (Fyfanon, 96% purity), whereas pure malathion (99% purity) was used in the present study. Commercial formulations may contain impurities such as isomalathion, a potent acetylcholinesterase inhibitor that also inhibits carboxylesterases involved in malathion detoxification. The presence of such impurities can enhance toxicity by limiting detoxification pathways (Jensen and Whatling 2010), potentially explaining the lower EC50 values reported in studies using formulated products.

Under ALAN exposure, a 48h EC50 could not be determined, as individuals appeared more resistant to malathion than those maintained under normal light conditions (Figure 1). A likely explanation for this pattern is the photolytic degradation of malathion under prolonged light exposure, which may have reduced the effective concentration available for uptake. Malathion toxicity depends on its bioactivation to malaoxon, a more potent acetylcholinesterase inhibitor formed through oxidative metabolism, whereas detoxification pathways involve rapid hydrolysis by esterases (Reed and Rubin 2014; Schopfer et al. 2025). Consequently, a reduction in malathion availability limits the formation of malaoxon and reduces overall toxicity. Consistent with this hypothesis, previous studies have shown that photolysis of pure malathion leads predominantly to the formation of non-acetylcholinesterase-inhibiting degradation products, resulting in a reduced toxic potential (Bavcon Kralj et al. 2007).

Alternatively, the reduced sensitivity to malathion under ALAN exposure may also involve stress-mediated physiological mechanisms. ALAN has been shown to increase the production of Reactive Oxygen species (ROS) in several organisms (Petrović et al. 2025; Eberhardt et al. 2025; Benedetto and Contin 2019). While excessive ROS accumulation can cause oxidative damage, moderate increases in ROS can act as signalling molecules that activate antioxidant and detoxification pathways (Li et al. 2021). In *Daphnia*, circadian regulation of oxidative balance plays a key role in physiological homeostasis. In 2020 a study demonstrated that alterations of light–dark cycles, including continuous or prolonged light exposure, significantly modify ROS accumulation and the activity and expression of antioxidant enzymes in *Daphnia pulex* (Cai et al. 2020). These results indicate that changes in illumination can disrupt circadian redox rhythms and induce compensatory antioxidant responses. Such stress-induced activation of antioxidant and detoxification pathways could potentially enhance the ability of *Daphnia magna* to cope with additional chemical stressors, including organophosphate insecticides such as malathion. Although this mechanism remains speculative in the present study, it provides a plausible explanation for the reduced sensitivity to malathion observed under ALAN exposure.

and highlights the need for future studies measuring oxidative stress markers and antioxidant responses under combined light and contaminant exposure.

During the sub-chronic experiment, malathion exposure did not significantly affect any of the measured endpoints. This absence of effect may result from the reduced sensitivity observed in the acute experiment, suggesting that the tested concentration ($0.2 \mu\text{g.L}^{-1}$) was insufficient to elicit measurable responses in our experimental design. Alternatively, malathion may have affected other endpoints not assessed in this study such as longevity or interclutch interval. In addition, malathion bioavailability may have been limited in our experiment system. The microalga used to feed *D.magna*, *Scenedesmus obliquus*, is known for its high bioremediation potential (Sui et al. 2025; Ummalyma et al. 2018). In particular, malathion has been shown to be biodegraded by certain microalgal species via carboxylesterase-mediated pathways, as demonstrated for *Chlorella sorokiniana* (Nanda et al. 2019). Moreover, biosorption may also have contributed to reducing malathion bioavailability. Microalgae possess functional groups on their cell wall capable of binding pollutants, thereby limiting their availability to other organisms (Akmukhanova et al. 2025). This mechanism has been reported for organophosphate insecticides such as chlorpyrifos (Habibah et al. 2020; Akmukhanova et al. 2025).

On the other hand, light pollution significantly affected most of the measured endpoints. Body size was affected at day 7 (Figure 2), with *Daphnia magna* exposed to ALAN being larger at this time point, whereas no difference was observed at day 14. This pattern suggests that ALAN may exert a transient effect during early developmental stages rather than long-term impacts on somatic growth. In contrast, an opposite temporal pattern was observed for eye size, with individuals exposed to ALAN exhibiting a slightly larger eye diameter at day 14 (Figure 4). This delayed response suggests that ALAN may differentially influence morphological traits, potentially reflecting alterations in developmental allocation or sensory investment later in life.

These results differ from several previous studies reporting a reduction in both body length and eye size under ALAN exposure (He et al. 2025; Li et al. 2022; Brandon and Dudycha 2014). In particular, Li et al. showed that ALAN reduced body length at the 6th instar (sexual maturity) and decreased eye diameter at the 10th instar, indicating cumulative negative effects of prolonged nocturnal illumination on somatic growth and sensory investment. However, Li et al. also emphasized that ALAN effects are strongly stage-dependent, with different traits responding at different developmental instars. In this context, our results similarly highlight the importance of developmental timing, as we observed early effects on body size and delayed responses in eye morphology, although the direction of the responses differed.

One possible explanation for these differences lies in indirect, bottom-up effects of ALAN mediated through changes in algal food resources. The lighting used in the present study was enriched in blue wavelengths which have been shown to stimulate the growth and lipid accumulation of *Scenedesmus obliquus* (Abomohra et al. 2019). Such wavelength-dependent metabolic changes in microalgae may increase food availability or nutritional quality for *D.magna* under ALAN exposure. In addition to potential changes in food availability or quality, ALAN may also directly influence the physiological capacity of *Daphnia* to exploit available resources. Cremer et al. (2022) showed that ALAN exposure increased the expression of genes involved in protease activity, suggesting enhanced digestive efficiency and nutrient assimilation (Cremer et al. 2022).

These morphological changes may have important functional consequences for reproductive performance. Consistent with this another study demonstrated that *D.magna* exposed to higher food concentrations (20,000 cells/mL instead of 5,000 cells/mL of algae) developed larger eyes, suggesting increased investment in visual structures under resource-rich conditions (Brandon and Dudycha 2014). Moreover, in 2015 they showed that increase eye diameter was associated with larger clutch sizes and consequently higher reproductive fitness in (Brandon et al. 2015). Because eye size is closely linked to visual capacity, these findings support the hypothesis that enhanced food availability or quality under ALAN exposure may promote increased sensory investment, which in turn could improve resource acquisition efficiency and reproductive output.

In line with this hypothesis, our results suggest that an increased reproductive output of *Daphnia magna* under ALAN exposure, with a 30% increase in the total number of neonates produced (Figure 6). This result should be interpreted with caution, as it may be partly explained by the earlier onset of reproduction observed under light exposure (Figure 5). An advanced reproductive timing could increase lifetime reproductive output by allowing individuals to produce a greater number of broods over the experimental period. Further analyses would be required to disentangle whether the observed increase in fecundity reflects a true enhancement of reproductive investment per brood or is primarily driven by changes in reproductive timing.

Importantly, previous work suggests that reproductive responses of *Daphnia* to ALAN may depend on population history (He et al. 2025). They showed that individuals originating from habitats chronically exposed to light pollution matured earlier and produced more offspring under ALAN conditions than individuals from light-naïve populations (He et al. 2025). The clones used in the present study was collected from a site located close to human habitation and within 100 m of a major road (N253), and may also be influenced by skyglow (Falcón et al.

2020) from nearby urban areas such as Brussels and Leuven. Such prior exposure to artificial light may therefore have contributed to the observed reproductive responses, highlighting the role of ecological history in shaping ALAN sensitivity.

Behaviourally, mean swimming activity of *Daphnia magna* was not significantly affected by either malathion or ALAN exposure (Figure 7). However, the absence of an effect on this specific endpoint does not imply that behaviour was unaffected overall. Previous studies have demonstrated that light pollution can alter other behavioural responses in *D.magna*, including diel vertical migration and phototactic behaviour (Moore et al. 2000; Tüzün et al. 2025). In addition, although clone-specific responses were not explicitly tested in the present study, exploratory data visualisation revealed variability among clones in their response to ALAN exposure, with some clones exhibiting higher activity levels than others. Such inter-clonal variability suggests that behavioural responses to ALAN may be genotype-dependent and that mean swimming activity alone may not capture more subtle or trait-specific behavioural alterations. This aligns with the study of (Maszczyk et al. 2021) which showed that behavioural response was clone specific.

5.Limitation and perspectives

A limitation of the present study is that malathion actual concentrations were not measured during the experiment, which prevented us from determining whether the differences observed in acute exposure were driven by chemical degradation (e.g. photolysis) or by direct organism-level responses. Future studies should include chemical analyses to quantify malathion concentrations over time and better assess its bioavailability under different light conditions and time.

In the acute experiment, a 48 h EC50 could not be estimated under ALAN due to the low immobilization observed across the tested concentration range. Future experiments should account for potential photodegradation by extending the concentration range, particularly for compounds known to be susceptible to photolysis.

In addition, measurements of algal biomass and food intake would help clarify whether the observed morphological and physiological changes in *Daphnia magna* resulted from altered resource availability or from direct effects of artificial light at night (ALAN) on the organisms.

Another limitation concerns the spectral composition of the light used in this study, which was enriched in blue wavelengths and may differ from natural nocturnal light or typical urban

skyglow. Future experiments should incorporate more environmentally realistic lighting scenarios, including different spectral compositions, light intensities, and temporal patterns (e.g. pulsed or fluctuating light regimes), as well as the use of commercial pesticide formulations rather than pure compounds to better reflect real-world exposure conditions.

Finally, extending the duration of the experiment would allow assessment of longevity, interclutch intervals, and later reproductive events. In the present study, many individuals produced only a single brood, limiting our ability to evaluate long-term reproductive strategies and fitness consequences under combined light and chemical stress.

6. Conclusion

In the present study, artificial light at night (ALAN) emerged as the primary driver shaping the responses of *Daphnia magna* under experimental stress conditions, whereas malathion exposure had limited detectable effects at the concentrations tested. Acute toxicity assays indicated a lower sensitivity to malathion than reported in previous studies, and this apparent reduction in toxicity under ALAN exposure likely reflects changes in malathion availability, such as photolytic degradation, as well as potential stress-mediated physiological responses.

Under sub-chronic exposure, malathion did not significantly influence the measured endpoints, possibly due to reduced bioavailability within the experimental system and biodegradation or biosorption by microalgal food sources. In contrast, ALAN significantly affected developmental trajectories, morphology, and reproduction, inducing transient increases in body size, delayed enlargement of eye diameter, and an overall increase in reproductive output. These effects appear to be mediated by a combination of indirect bottom-up mechanisms, through changes in algal resource availability and quality, and direct physiological responses, including enhanced digestive capacity.

However, the absence of direct measurements of malathion concentrations limited our ability to disentangle chemical degradation from organism-level tolerance mechanisms and to assess interactions between ALAN and malathion under sub-chronic exposure. Future studies combining chemical analyses with longer-term life-history assessments will be essential to better understand how light pollution interacts with chemical stressors. Given the central role of *Daphnia* and zooplankton more broadly as key intermediates between primary producers and higher trophic levels, ALAN-induced changes in their performance may propagate through freshwater food webs and contribute to ecosystem-level imbalances. Overall, our findings

highlight ALAN as a key environmental driver capable of modulating organismal sensitivity to contaminants in freshwater ecosystems.

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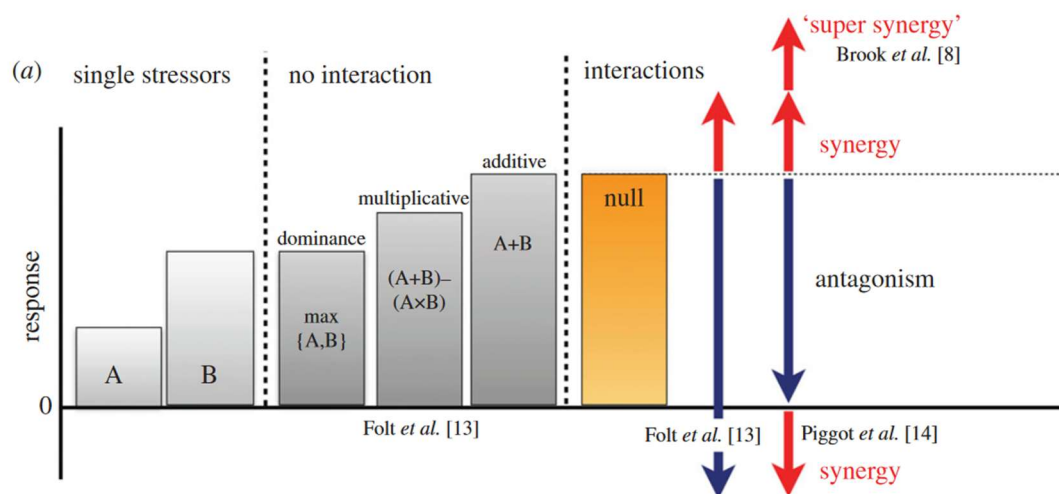
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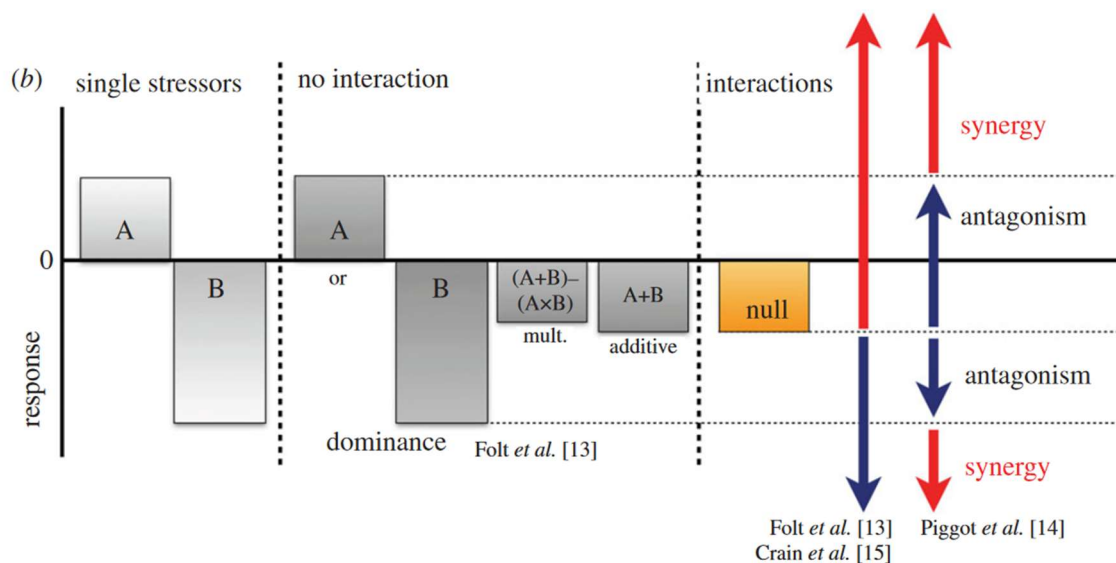
8. Annex

Annex 1: Multiple stressors



Annex 1: Schematic representation of possible interactions between multiple stressors (A et B), when these stressors “impact a biological response in the same direction when acting separately” (Côté *et al.* 2016).

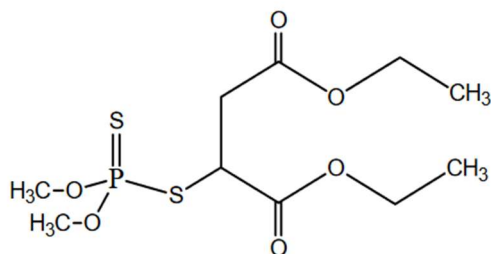
Annex 2: Multiple stressors with opposing effect



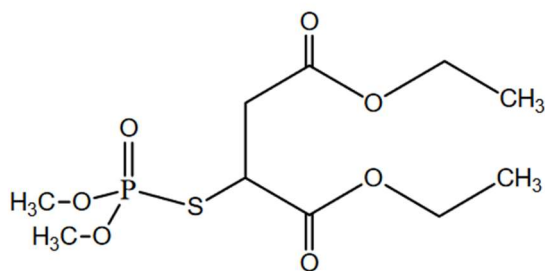
Annex 2: Schematic representation of possible interactions between multiple stressors (A et B), when these stressors have opposing effects on a biological response (Côté *et al.* 2016).

Annex 3: Chemical structure

Chemical Structure of Malathion



Chemical Structure of Malaoxon



Annex 3: Chemical structure of malathion and malaoxon (Newhart 2006)

Annex 4: Light composition of culture lamp (Philips MASTER TL-D 36W/840)



Annex 4. Spectral power distribution (left panel) and photometric characteristics (right panel) of the light source used in the *Daphnia magna* culture room, measured with a handheld spectroradiometer (UPRtek, PG200N). The spectrum shows a dominant peak in the blue region with additional emissions in the green–yellow range. Photometric measurements indicate a correlated colour temperature (CCT) of 4072 K, a colour rendering index (CRI) of 92.2, and an illuminance of 2105 lux.

Annexe 5: ADaM medium

The ADaM medium was prepared using three stock solutions and sea salt at the concentrations indicated in Table X, all dissolved in Milli-Q water. Stock solutions were prepared separately and stored at 4 °C. All preparation steps were performed under a laminar flow hood to minimize the risk of contamination.

The final medium was obtained by adding the appropriate volumes of stock solutions A, B, and C, together with sea salt, to the required volume of water (Annex 5.1).

Stock solution A contained $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ at a concentration of 117.6 g L^{-1} , stock solution B contained NaHCO_3 at 25.2 g L^{-1} , and stock solution C contained SeO_2 at 0.07 g L^{-1} .

Milli-Q water (L)	Sea salt (g)	Stock solution A (ml)	Stock solution B (ml)	Stock solution C (ml)
10	3.33	23	22	1
50	16.6	115	110	5
60	19.9	138	132	6

Annex 5.1. . Composition and preparation of the ADaM medium. The table shows the volumes of stock solutions A, B, and C and the amount of sea salt required to prepare different final volumes of ADaM medium, as well as the chemical composition and concentrations of each stock solution.

Annex 6: *Scenedesmus obliquus* culture and maintenance

Scenedesmus obliquus (also known as *Tetradesmus obliquus*) was cultured in 3N-BBMV medium (recipe provided in Annex 6.1). Cultures were maintained in the Daphnia culture room under a 16:8 h light–dark photoperiod at a temperature between 19 and 21 °C. Cultures were refreshed every 10 days by transferring 20 mL of algal culture into 400 mL of fresh medium.

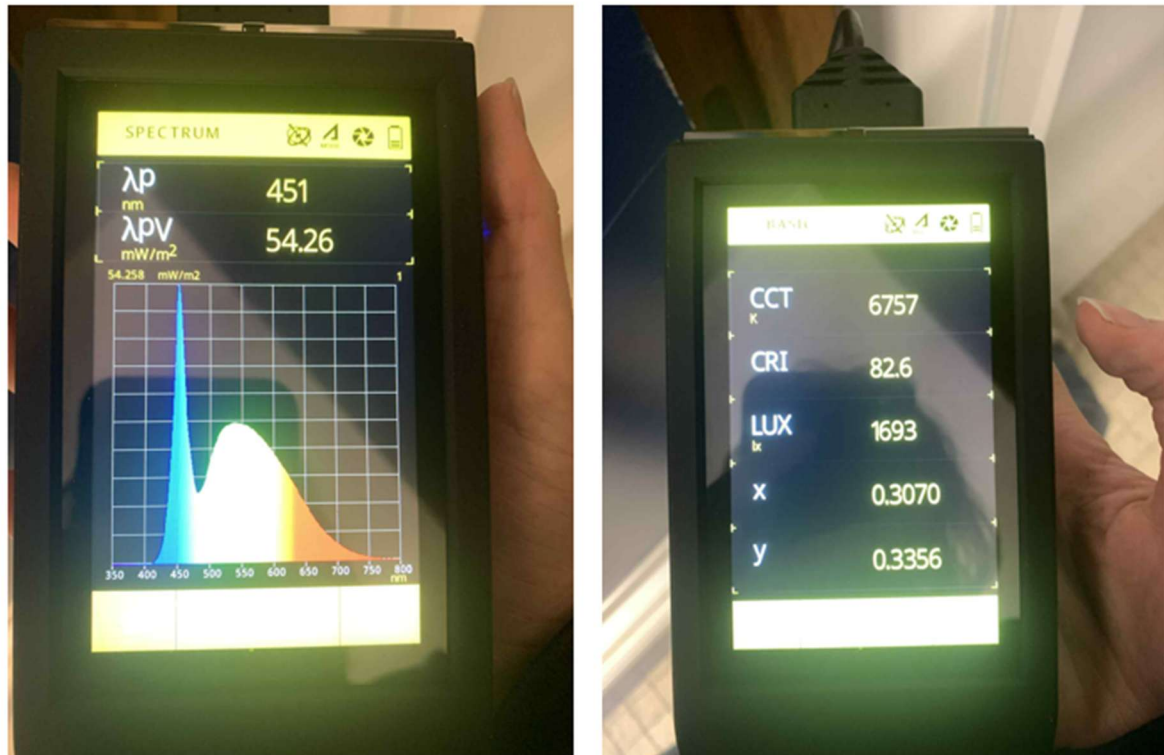
The remaining algal suspension was stored at 4 °C and allowed to settle by decantation. After decantation, approximately one quarter of the supernatant was removed, and the optical density (OD) of the remaining suspension was measured at 800 nm using a spectrophotometer. If the OD was equal to 2.1, the algal suspension was stored at 4 °C until use for feeding *Daphnia magna*. If the OD was lower than 2.1, ADaM medium was added to adjust the suspension to the desired optical density.

		mg/L	mg/200 mL	g/200 mL		
macro nutrients	CaCl ₂ .2H ₂ O	25	5000	5	Each macro nutrient in 6 separated 200 ml bottles	stored à 4°C
	K ₂ HPO ₄	75	15000	15		
	KH ₂ PO ₄	175	35000	35		
	MgSO ₄ .7H ₂ O	75	15000	15		
	NaCl	25	5000	5		
	NaNO ₃	750	150000	150		
micro nutrients in g/L				All micro nutrients can be mixed in one 1L bottle. Respect the order, start with Na2.EDTA.		
micro nutrients	Na ₂ .EDTA	4,500			4,500	
	FeCl ₃ .6H ₂ O	0,582			0,582	
	MnCl ₂ .4H ₂ O	0,246			0,246	
	ZnCl ₂	0,030			0,030	
	CoCl ₂ .6H ₂ O	0,012			0,012	
	Na ₂ MoO ₄ .2H ₂ O	0,024		0,024		
vitamins	B1	0,010000 0			Mix, filtered (0,22µm) and aliquoted by	stored à -20°C
	H	0,000002 5				

	B12	0,000001 5				
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Annex 6.1. Composition of the 3N-BBMV culture medium showing macro-nutrient, micro-nutrient, and vitamin stock solutions and their preparation conditions.

Annex 7: Light spectral composition of experimental set up



Annex 7.1. Spectral and photometric characterization of the ClassicLED G2 light source used in the incubators. The left panel shows the emission spectrum of the LED light, with a peak wavelength around 451 nm, measured using a spectroradiometer (UPRtek, PG200N). The right panel shows photometric parameters of the light source, including correlated colour temperature (CCT), colour rendering index (CRI), and chromaticity coordinates (x, y), measured at the level of the experimental containers. Night-time illumination under artificial light at night (ALAN) was provided by the same lamp model and identical spectral and photometric characteristics, with only the light intensity reduced to 20 lux.

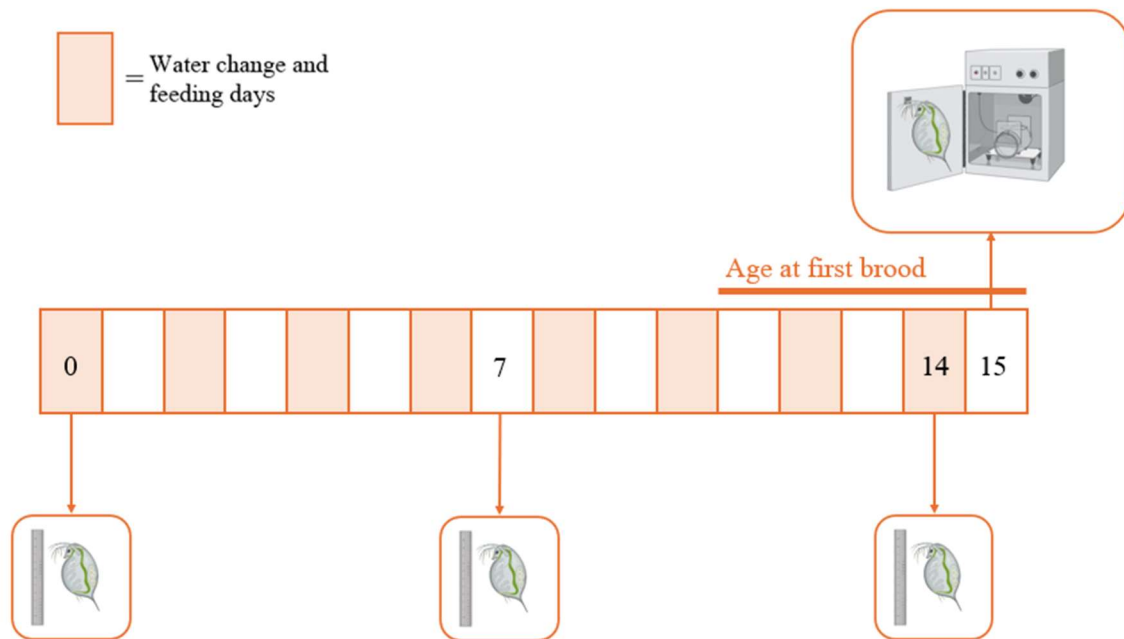
Annex 8: Experimental design

Concentrations ($\mu\text{g. L}^{-1}$)		
0		
0.9		
1.15		
1.45		
1.8		
2.25		
2.8		
3.5		

Annex 8.1. Experimental design of the acute malathion exposure under contrasting light conditions. *Daphnia magna* neonates (< 24 h old) were exposed for 48 h to a gradient of malathion concentrations (0, 0.9, 1.15, 1.45, 1.8, 2.25, 2.8 and 3.5 $\mu\text{g L}^{-1}$) under two light regimes: a natural light–dark cycle (16 h light : 8 h dark; left column) and artificial light at night (ALAN; continuous low-intensity illumination during the night; right column). For each concentration and light treatment, four replicate tubes were used, each containing five individuals. Immobilization was assessed after 24 h and 48 h of exposure.



Annex 8.2. Experimental design of the sub-chronic exposure. Neonates of *Daphnia magna* (<24 h old) were individually reared for 15 days under two light regimes: a control photoperiod with a natural light–dark cycle (left panel) and artificial light at night (ALAN; right panel). Individuals were exposed either to control medium or to malathion (0.2 $\mu\text{g L}^{-1}$, in shaded area). Each treatment combination included 10 replicates and 3 clonal lineages (DM7, DM9, DM22)



Annex 8.3. Timeline of the sub-chronic experiment. Neonates of *Daphnia magna* (<24 h old) were individually exposed to the experimental treatments for 15 days. Body size and eye diameter were measured at days 0, 7, and 14. Reproductive onset (age at first brood) was monitored daily throughout the experiment, with individuals that did not reproduce considered right-censored. Swimming behaviour was assessed at day 15. Shaded boxes indicate days on which water renewal and feeding were performed.

Annex 9 Package R

Package	Analysis	Use
readxl	All	Import raw experimental data from Excel files into R
drc	Acute	Import raw experimental data from Excel files into R
car	All	Fit dose–response models and estimate EC50 values for malathion exposure, Analysis of deviance (Type II Wald χ^2 tests)
cowplot	Acute	Combine and arrange ggplot figures into multi-panel layouts
ggplot2	All	Graphic visualisation
MuMin	All	Combine and arrange ggplot figures into multi-panel layouts
dplyr	All	Data cleaning, filtering, and manipulation prior to statistical analyses
tidyr	All	Reshape and organise data into tidy format suitable for modelling
lme4	Size, Behaviour, Probability of reproduction	Fit linear and generalized linear mixed-effects models (LMMs, GLMMs)

emmeans	Size, Fecundity	Post hoc comparisons and estimation of marginal means
coxme	Age at first brood	Fit mixed-effects Cox proportional hazards models
survival	Age at first brood	Kaplan–Meier estimation and Cox proportional hazards models
glmmTMB	Fecundity	Fit negative binomial and zero-inflated mixed models
survminer	Age at first brood	Visualisation of survival curves and log-rank tests
DHARMa	All GLMMs	Simulation-based residual diagnostics
lmerTest	Size, Behaviour	p-values and degrees of freedom for linear mixed-effects models

Annex 9. Overview of R packages used in this study, their associated analyses, and their primary functions.