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Fluence rate or cumulative dose? Vulnerability of larval northern pike (*Esox lucius*) to ultraviolet radiation[‡]

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Abbreviations: PAR, photosynthetically active radiation; TOC, total organic carbon; UVA, ultraviolet A; UVB, ultraviolet B; UVC, ultraviolet C; UVR, ultraviolet radiation;

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ABSTRACT

Newly hatched larvae of northern pike were exposed in the laboratory to four fluence rates of ultraviolet radiation (UVR; **290 – 400 nm**) over three different time periods, resulting in total doses ranging from 3.0±0.2 to 63.0±4.4 kJ/m^2. Mortality and behavior of the larvae were followed for 8 to 12 days, and growth measured at the end of the experiment. Also, the principle of reciprocity – that the UVR induced mortality depends on the cumulative dose, independent of fluence rate – was tested. Fluence rates higher than 1480±150 mW/m^2 caused mortality and growth retardation. The highest fluence rate (3040±210 mW/m^2) caused 100% mortality in 5 days. All fluence rates caused behavioral disorders, which led to death at fluence rates higher than 1480 mW/m^2. **Reciprocity failure occurred with the lowest and highest dose (550±45 and 3040±210 mW/m^2, respectively).** The results show that fluence rate is of primary importance when assessing the UVR-related risk.

INTRODUCTION

Ultraviolet radiation (UVR; **290 – 400 nm**) has been shown to be detrimental to fish, especially in the embryo and larval stages (1-9). However, there are very large differences between species. It was recently shown that larvae of northern pike (*Esox lucius*) are highly sensitive even to the levels of UVR encountered currently in early summer (10).

In Fennoscandia, pike spawns in April-May in shallow waters (depth less than 1 m) at temperatures around 5 – 7 ^{o}C with vegetation as the spawning base. The fertilized eggs swell and adhere to plants, which prevents them from sinking down to the bottom. Newly hatched larvae attached to plants remain mostly nonmotile for the first few days of their lives, and are neither negatively nor positively phototactic. Freely swimming larvae are positively phototactic and often swim very near the water surface, where they may become exposed to UVR (11-12). The daily exposure may vary markedly depending on, e.g., weather, **water clarity and hydrographic conditions** (13, 14). Due to stratospheric ozone depletion, UVB radiation (280 - 315 nm) may increase up to 50 % in May in mid-boreal regions (15). To assess the risk of enhanced UVB radiation to larval pike, it is important to know whether the fluence rate or the cumulative dose over a longer period determines the biological effects.

The law of reciprocity, first presented by Bunsen and Roscoe in the 1850s, states that photochemical reactions depend only on the total absorbed energy (cumulative dose), and are statistically independent of the two factors that determine total absorbed energy, i.e., fluence rate (radiant intensity) and exposure time (16). The law of reciprocity holds for most biological systems and endpoints that have been studied when reciprocity has not been

compromised by repair mechanisms (2, 5, 16, 17, 18). This has been shown also in fish: Reciprocity held in experiments where exposures were short and fluence rates and doses were high (5) but failed in experiments with long exposures and low fluence rates (2).

The issue of reciprocity is important because it gives knowledge on how UVR affects fish. Moreover, extrapolating from laboratory experiments to field conditions is impossible without reciprocity. Construction and application of dose-dependent biological weighting functions can only be done if reciprocity holds for the organism (17-19).

On the basis of the facts stated above, the aim of this study was to determine the tolerance limits of pike larvae, and to test the principle of reciprocity, hypothesizing that the UVR-induced mortality and the preceding sublethal effects would depend on total cumulative dose received, independent of fluence rate.

MATERIALS AND METHODS

Animals and water characteristics. The fertilized eggs of pike (progeny of three females and one male) were obtained from the municipality of Jämsä (Timo Paajoki, Lake Päijänne, 61^{o}24 N, 25^{o}24 E) in late May, 2003. Once transferred to the wet laboratory of the University of Jyväskylä, the eggs were incubated and hatched in flow-through hatchery cones at 10 ^{o}C. The photoperiod during this acclimation phase was 12h:12h. The water characteristics of the lake and the wet laboratory are presented in Table 1.

<Table 1>

Exposure system and sampling. UV radiation was provided in the laboratory using a fluorescent polychromatic lamp (UVB-313, Q-Panel, Cleveland, OH, USA). Any possible ultraviolet C (UVC; under 280 nm) was blocked with a cellulose diacetate filter (Clarifoil, Derby, UK). The spectra **of the four fluence rates** are shown in Fig. 1. Control larvae received visible light only (TLD 36 W/950 daylight, Philips, Eindhoven, Netherlands). Visible light was present also in the UV treatment. The photoperiod was 12h:12h, the same as during the acclimation phase before hatching. UV was quantified using a Hamamatsu Photonic Multichannel Spectral analyser (model PMA-11), which measured the wavelength area 280 – 380 nm at every 1 nm. The device was calibrated the same spring against the sun simulator run by the Finnish Meteorological Institute (Jokioinen, Finland).

<Figure 1>

Newly hatched (< 24 h) larvae were carefully transferred to 1-l Pyrex glass bowls filled with water from the hatchery. Larvae were exposed to UVR once for 1.5, 3 or 6 hours at midday. There were four fluence rates and one UVR-free control group (visible light only). **The cumulative dose is defined as the fluence rate multiplied by exposure time**, thus there were 12 UVR doses ranging from 3.0±0.2 to 63.0±4.4 kJ/m^2 (mean±variation). The UVR fluence rates and doses are presented in Table 2. Each treatment was replicated three times,

with 40 larvae in each group. After the UVR episodes all the larvae received visible light only (TLD 36 W/950 daylight, Philips).

<Table 2>

After UVR exposure, mortality and behavior were monitored twice a day by gently blowing water with a pipette on each larva to make it swim. Completely non-motile larvae were designated as dead. Larvae spinning around, otherwise unable to swim straight, or just barely moving, were designated as behaviorally abnormal. **The animals were sampled for growth analysis when they had used up more than ¾ of their yolk sac contents (1.5 h and 3 h UVR treatment). Alternatively, the experiment was finished when all UVR-irradiated larvae showed severe behavioral symptoms (6 h UVR treatment).**

Growth Measurements. For the 1.5 and 3 h exposures to UVR, the total length of all viable larvae and the size of the fry sac of survivors were determined with the help of a microscope at the end of the experiment. At the same time, the behavior of the larvae was examined and the animals from each replicate were sorted into two groups of normally and abnormally behaving (= unable to swim straightforward) larvae. Because of low survival there were three to five larvae in each group. **No sampling was conducted in the 6 h exposure group because the experiment was finished before any changes in growth could be seen.**

Statistics. Statistics were performed with SPSS 10.0. The effect of dose on daily mortality and behavior were tested with ANOVA for repeated measures and Dunnett's T3 test. The effect of

cumulative UVR dose on growth was tested with ANOVA and Dunnett's T3 test. To test reciprocity, the effect of fluence rate on daily mortality was tested with multivariate ANOVA, with dose as a covariate (Analysis of covariance, ANCOVA, in SPSS). Time to 50 % mortality was taken from the observed mortality data in all treatments where 50 % mortality was reached. It was estimated with Probit analysis (SPSS) for the treatment with 15.7±1.6 kJ/m^2.

RESULTS

Mortality

Figure 2 shows mortality as a function of time after irradiation. **Irradiation with the highest fluence rate led to 100% mortality in 100 hours even with the shortest irradiation times (1.5 h, Fig. 2a; and 3 h, Fig. 2b). The lowest fluence rate (550±45 mW/m^2), on the other hand, did not cause mortality except very low and late mortality with the longest irradiation time (6 h, total cumulative dose 11.5±0.8 kJ/m^2, Fig. 2c). Although mortality caused by the irradiation with 11.5±0.8 kJ/m^2 was statistically significant from control values at 162 and 182 hours after irradiation (ANOVA, $p < 0.05$), the overall mortality of this treatment, tested with ANOVA for repeated measures, did not differ from the control value ($p > 0.05$).**

<Figure 2>

Reciprocity

Reciprocity - i.e. the direct relation between total cumulative dose received and mortality - was examined by testing the effect of fluence rate on mortality with dose as a covariate. **When all the fluence rates were included, both fluence rate and dose contributed to mortality (F = 37.767, df = 14, *P* < 0.001, Pillai's Trace), and thus reciprocity did not hold for all the fluence rates tested.** When both control (no UVR), and the lowest UVR fluence rate (550±45 mW/m^2) were omitted, the fluence rate continued to contribute **to mortality (F=3.584, df=16, *P* = 0.007). Also dose contributed to mortality (F=24.204, df=8, *P* < 0.001).** When the two intermediate fluence rates (1480±150 and 2200±130 mW/m^2) alone were tested, the effect of fluence rate was not significant (**F = 0.641, df = 2,** *P* = 0.732). **The time to 50% mortality for each treatment is shown in Figure 3.**

<Figure 3>

Behavioral Abnormality

All the UV fluence rates caused spiral swimming, i.e., the inability of the larva to swim straight ahead (Fig. 4). Although recovery at the lowest total doses (3.0±0.2 and 5.9±0.4 kJ/m^2) given at the lowest fluence rate (550±45 mW/ m^2) (Fig. 4a, b) was almost complete, at all the other doses spiral swimmers showed 100% mortality.

<Figure 4>

Growth

Of the larvae designated as normal at the end of the experiment, the control larvae were longer than the irradiated ones (ANOVA, F = 6.755, *P* < 0.001), except those irradiated with the lowest dose of 3.0±0.2 kJ/m^2. There were no differences in the size of yolk sacs (ANOVA, F = 1.0, *P* = 0.445). Abnormally behaving larvae were shorter and possessed more yolk than normally behaving ones (ANOVA, $P < 0.001$). The larvae that had been exposed to UVR - except the lowest total dose (3.0±0.2 kJ/m^2) - were shorter (ANOVA, $P < 0.001$) (Fig. 5a) and possessed more yolk (ANOVA, $P = 0.001$) than controls (Fig. 5b). The results of pairwise comparisons (Dunnett's T3) are shown in Fig 5.

<Figure 5>

DISCUSSION

The answer to the question asked in the **title** of this article is "both". Both the fluence rate and the total dose matter. Fluence rate plays a big role when it is very low or very high: the lowest fluence rate (550±45 mW/m^2) caused much less mortality than expected from the total dose received, and this response started very late compared to similar doses given at higher fluence rates. Recovery from behavioral disorders also occurred at the lowest fluence rate. Irradiation at the highest fluence rate (3040±210 mW/m^2) always led to complete mortality within 5 days.

The principle of reciprocity has been evaluated for some aquatic species, with inconsistent results. In marine zooplankton and ichthyoplankton reciprocity holds for cod (5) and **copepod *Calanus finmarchicus* (18)**, and does not hold for northern anchovy (1, 2), shrimp *Pandalus platyceros* (20) nor euphausid *Thysanoessa raschii* (21). In freshwater organisms reciprocity holds for rotifer *Asplancha girodi* (17) and copepod *Boeckella gracilipes* (22-24) but not for cladocerans *Daphnia* spp. (17, 25) and *Ceriodaphnia dubia* (23), mayfly *Diphetor hageni* nor chironomid *Corynomeura taris* (26).

In non-biological systems failure of the reciprocity law commonly occurs with both very low and very high fluence rates (16). Similarly, in our study reciprocity clearly failed with low and high fluence rates. Our results are both novel and in accordance with former studies on fishes: with a broad range of doses and fluence rates, and with relatively long exposures (4 to 12 days), reciprocity did not hold in larval northern anchovy (2). However, reciprocity held for cod embryos when exposures were short (1 – 5 hours) and fluence rates high (5). It was hypothesized by Kouwenberg et al. (5) that reciprocity holds when damage dominates repair, i.e. when repair processes do not significantly compromise reciprocity (short and intense exposures). When repair dominates (low fluence rates, long exposures), reciprocity fails. Grad et al. (17) have hypothesized and partly proven that photoenzymatic repair (PR) may compromise reciprocity in zooplankton. This may be the case with the low fluence reciprocity failure in pike, but to confirm this, the PR capacity of northern pike should be investigated in detail.

The model proposed by Martin et al. (16) suggests that high fluence reciprocity failures (both in non-biological and biological systems) result from a different action mechanism, as described in the band theory. The band theory is generally accepted and applied to biological

systems such as photosynthesis, biochemical processes and human vision. Biological and medical photoresponse data used by Martin et al. also support its suitability in biological reciprocity experiments (16). In short, the band theory states that in a solid the energy states of electrons tend to cluster into energy bands separated by non-allowed energy levels (energy gaps). Electrons can be excited into another band only if the absorbed electron has sufficient energy to allow the electrons to jump the energy gap. The excited electrons can then either return to their former energy band, or become trapped. As the radiant flux increases, the amount of excited electrons increases as does the amount of trapped electrons. When all the traps inside the material become full the electrons can only return to the ground state, and this leads to discontinuity in the effect of radiation (16). In a biological system like fish larvae, these “traps” could be parts of biological molecules acting as electron scavengers. When the UVR fluence rate is very high the scavengers become “filled” with electrons, and much more damage to other biological molecules occurs than expected. This damage is then manifested in higher mortality than expected.

Pike larvae were vulnerable to very low levels of UVR. It must be taken into account, however, that the ratio of UVB:UVA:PAR was skewed in our system and does not represent the conditions in nature. Further, the low levels of UVA and PAR in our system most likely attenuated any photoenzymatic repair, which may exaggerate the effects of UVR in our study. In a field situation, with similar UVB fluence rates as the two lowest ones in the laboratory, less mortality was seen in larval northern pike than in the present study (13). Another reason for the difference between laboratory and field may be that the water in the laboratory (TOC 2.0 - 2.5 mg/l) was more transparent to UVR than the water in the study lake (TOC 6.5 - 8.1

mg/l). However, there are lakes in Fennoscandia that have even higher transparency to UVR than the water in the laboratory.

Larval whitefish and vendace are much more tolerant of UVR than pike (27). UVR caused no mortality in whitefish and vendace even after 14 days of exposure (3 hours daily) (20), whereas one single exposure caused considerable mortality in pike in this experiment. Of marine species living in the same latitudes, larval cod (*Gadus morrhua*) also seems to be much more tolerant of UVR than larval pike: cod larvae irradiated with 1.43 W/m^2 (unweighted) UVB did not reach 50% mortality in 7 days (5) whereas for pike larvae the highest fluence rate of 1.7 W/m^2 (unweighted) UVB resulted in 100% mortality in 5 days.

Larvae in all the present UV treatments developed spiral swimming, i.e. the inability of the larva to swim straight ahead. The larvae exposed to the lowest fluence rate (550±45 mW/m^2) and shortest irradiation times (total doses 3.0±0.2 and 5.9±0.4 kJ/m^2) showed recovery. In a previous experiment the spiral swimming condition always led to mortality (10); however, the fluence rates in that experiment were higher than the lowest fluence rate in this one. Thus there is a threshold near the fluence rate of 550±45 mW/m^2 and dose of **around 6 kJ/m^2 below which fish can recover**.

A single small dose of UVR attenuated the growth of pike larvae, even when the recovery from the behavioral syndrome occurred (at 5.9±0.4kJ/m^2); thus growth seems to be the most sensitive of the variables studied in these experiments. It has been observed previously that UVR affects the growth of embryos and larvae of marine species, e.g. northern anchovy (*Engraulis mordax*), pacific mackerel (*Scomber japonicus*), and plaice (*Pleuronectes platessa*) (1, 7) and freshwater Cichlid fish *Cichlasoma nigrofasciatum* (28). Larval growth of freshwater fish whitefish and vendace was not affected by UVR (27).

Some of the retardation in pike growth resulted from inability to absorb yolk, but some was not associated with differences in yolk metrics. UVR retards the yolk absorption of larvae of Northern anchovy, Pacific mackerel and sockeye salmon (1, 6). Both the inability to absorb yolk and growth retardation without any difference in yolk absorption can arise from DNA damage caused by UVR in pike (Vehniäinen, unpublished results). The reason for the retardation of growth following UVR-induced DNA damage may either be the energy costs of repair - because the yolk represents a predetermined amount of energy that can be used either for tissue growth or respiration, increased metabolism will result in a decrease of growth (29) - or to cell cycle arrest before complete repair.

A very contrasting dicotomy was seen with shortest irradiation times (1.5 h and 3 h) and intermediate fluence rates of 1480±150 and 2200±130 mW/m^2 (total doses 8.0±0.8 – 23.2±1.4 kJ/m^2). At these fluence rates some of the larvae were not affected behaviorally at all, showing normal ability to absorb yolk, while others developed severe spiral swimming syndrome with apparent inability to absorb their yolk, and eventually died. These dose rates constitute a threshold level at which some larvae are capable of dealing with the stress caused by UVR and some are not.

Our results show that fluence rate is always of crucial importance when assessing the risk caused by UVR. Experiments concerning the effects of increasing UVB radiation have to be done with the help of artificial light sources. Comparing only total doses or cumulative daily doses to doses occurring in nature nowadays or in the future can be misleading if the total dose is attained with excessively short irradiation times and with fluence rates not occurring in nature. Moreover, there are differences between species in what fluence rates and

doses are tolerable, and therefore very likely also in the ranges of fluence rates at which reciprocity holds.

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FIGURE LEGENDS

Figure 1. Spectra of the lamps used in the experiments. The unweighted fluence rates (mean±variation): a = 550±45 mW/m^2, b = 1480±150 mW/m^2, c = 2200±130 mW/m^2, and d = 3040±210 mW/m^2.

Figure 2. Survival of pike larvae as a function of time after irradiation. **Symbols mark different fluence rates,** for total doses, see Table 2. a) Larvae irradiated for 1.5 hours. b) Larvae irradiated for 3 hours. c) Larvae irradiated for 6 hours. Bars represent standard deviation from the mean. N = 3 x 40 larvae per treatment.

Figure 3. **Time to 50% mortality of pike larvae as a function of total UVR dose. Time to 50 % mortality estimated with Probit analysis (SPSS) for the treatment with 15.7±1.6 kJ/m^2 (bars represent 95% confidence limits) and taken from the mortality data in all other treatments, where 50 % mortality was reached (bars represent limits where 50% mortality was reached in none and in all of the three replicates).**

Figure 4. Behavior of pike larvae as a function of time after irradiation. **Symbols mark different fluence rates,** for total doses, see Table 2. Normally swimming % is calculated from all larvae in a replicate (40) by subtracting dead and abnormally swimming ones (see text). For total doses, see Table 2. a) Larvae irradiated for 1.5 hours. b) Larvae irradiated for 3 hours. c) Larvae irradiated for 6 hours. Bars represent standard deviation from the mean. N = 3 x 40 larvae per treatment.

Figure 5. Growth response of pike larvae to UV radiation. Groups marked with the same letters do not differ significantly from each other (ANOVA, Dunnet's T3 test, $p>0.05$) a) Length and b) Size of yolk sac of pike larvae irradiated with different total doses of UVR, measured 11 days after irradiation. Bars represent standard deviation from the mean.

Table 1. Water characteristics in Lake Päijänne (annual ranges), from which the fish eggs were obtained, and Jyväskylä University wet laboratory where they were incubated until hatch and threrafter. Data of Lake Päijänne from the regional environment center of Central Finland.

	Lake Päijänne	Jyväskylä university wet laboratory
pH	6.2 – 7.3	7.4 – 7.7
conductivity (mS/m)	5.8 – 7.8	24.6
total organic carbon (TOC) (mg/l)	6.5 – 8.1	2.0 – 2.5

Table 2. The experimental design showing the fluence rates and the total doses in the experimental treatments. Newly hatched pike larvae were exposed to UV in Pyrex glass bowls,

40 larvae in each bowl. Each treatment was replicated three times (N = 3 x 40). Larvae were irradiated once at four fluence rates for 1.5, 3 or 6 hours.

Fluence rate (mW/m^2)	dose in 1.5 h (kJ/m^2)	dose in 3 h (kJ/m^2)	dose in 6 h (kJ/m^2)
550±45	3.0±0.2	5.9±0.4	11.5±0.8
1480±150	8.0±0.8	15.7±1.6	30.8±3.2
2200±130	11.9±0.7	23.2±1.4	45.6±2.8
3040±210	16.4±1.1	34.1±2.2	63.0±4.4

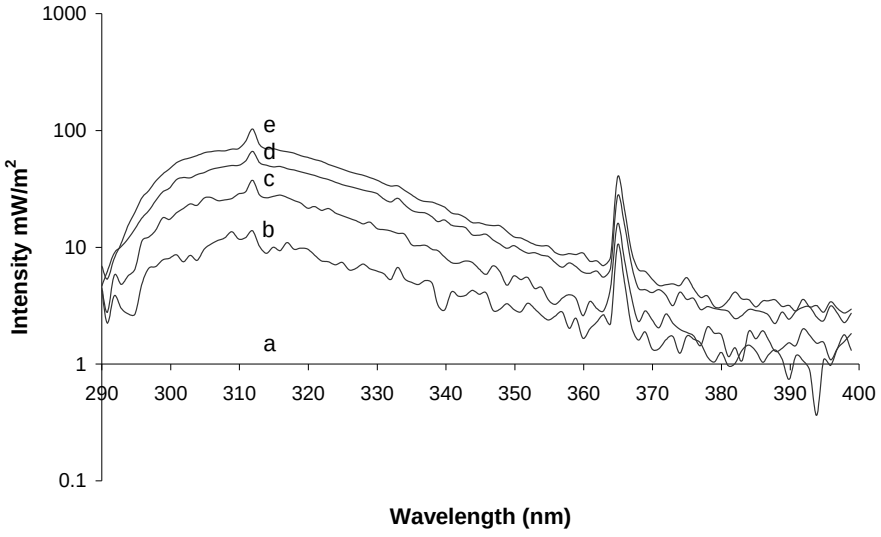


Figure 1.

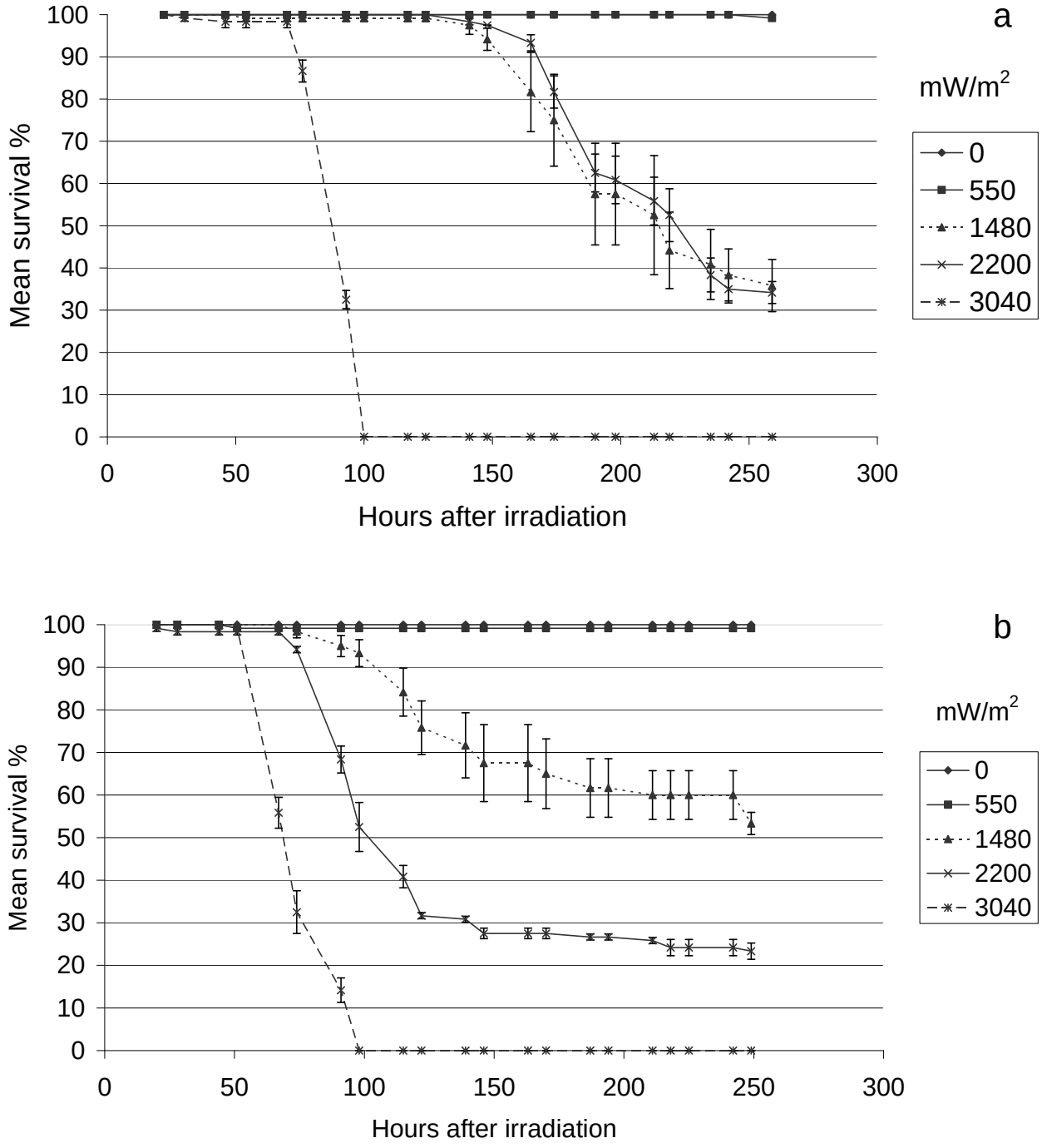

a
mW/m2
0
550
1480
2200
3040
Mean survival %
Hours after irradiation
b
mW/m2
0
550
1480
2200
3040
Mean survival %
Hours after irradiation

Figure 2.

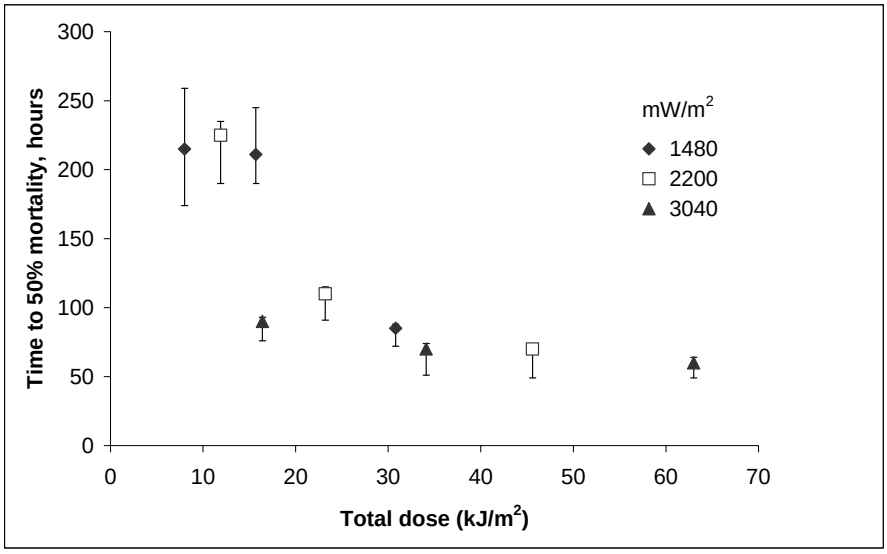
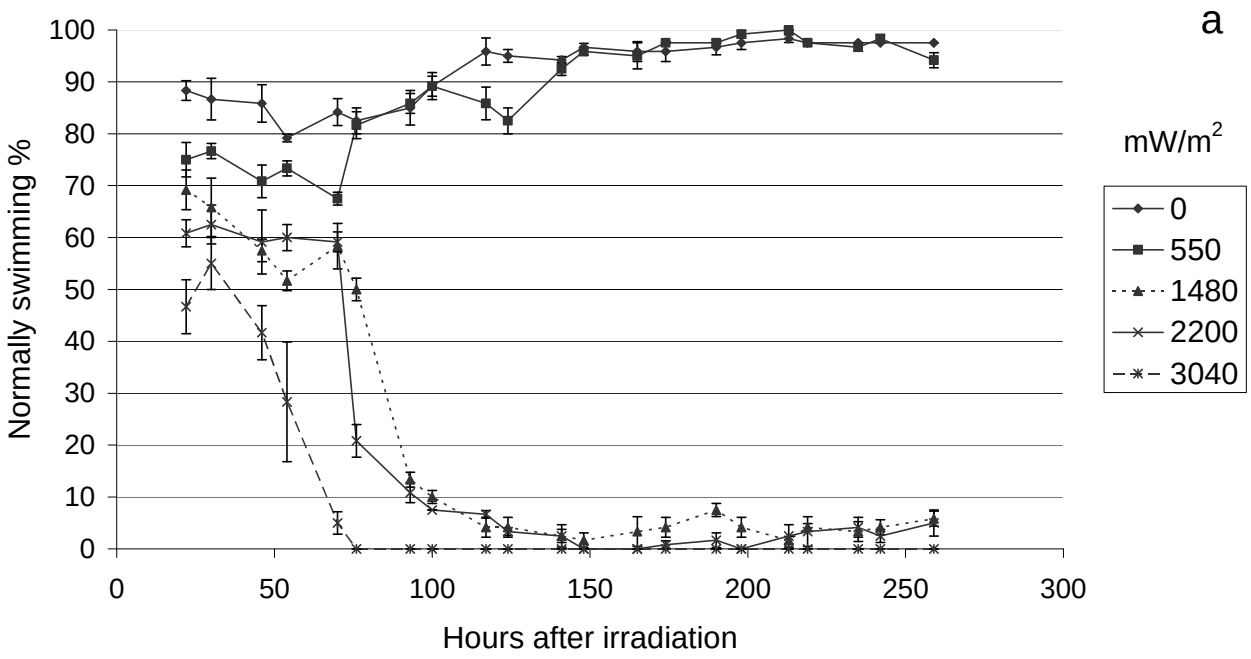
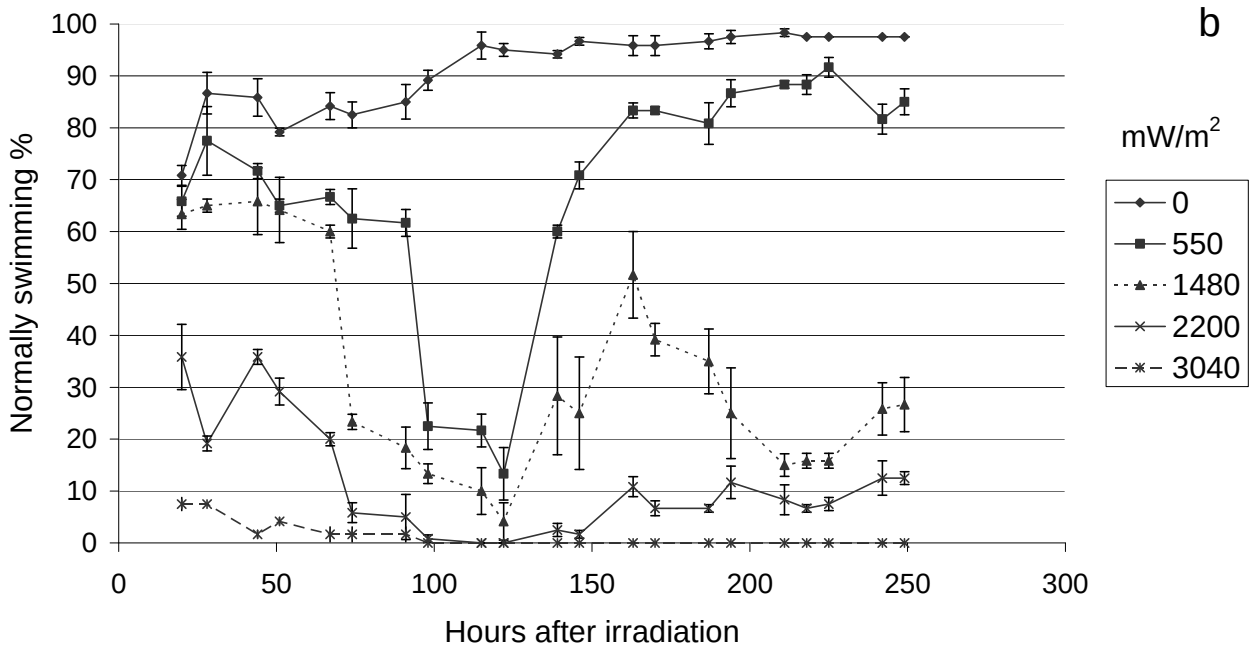

Time to 50% mortality, hours
300
250
200
150
100
50
0
0
10
20
30
40
50
60
70
Total dose (kJ/m2)
mW/m2
1480
2200
3040

Figure 3.


a
Normally swimming %
100
90
80
70
60
50
40
30
20
10
0
0
50
100
150
200
250
300
Hours after irradiation
mW/m2
0
550
1480
2200
3040


b
Normally swimming %
Hours after irradiation
mW/m2
0
550
1480
2200
3040

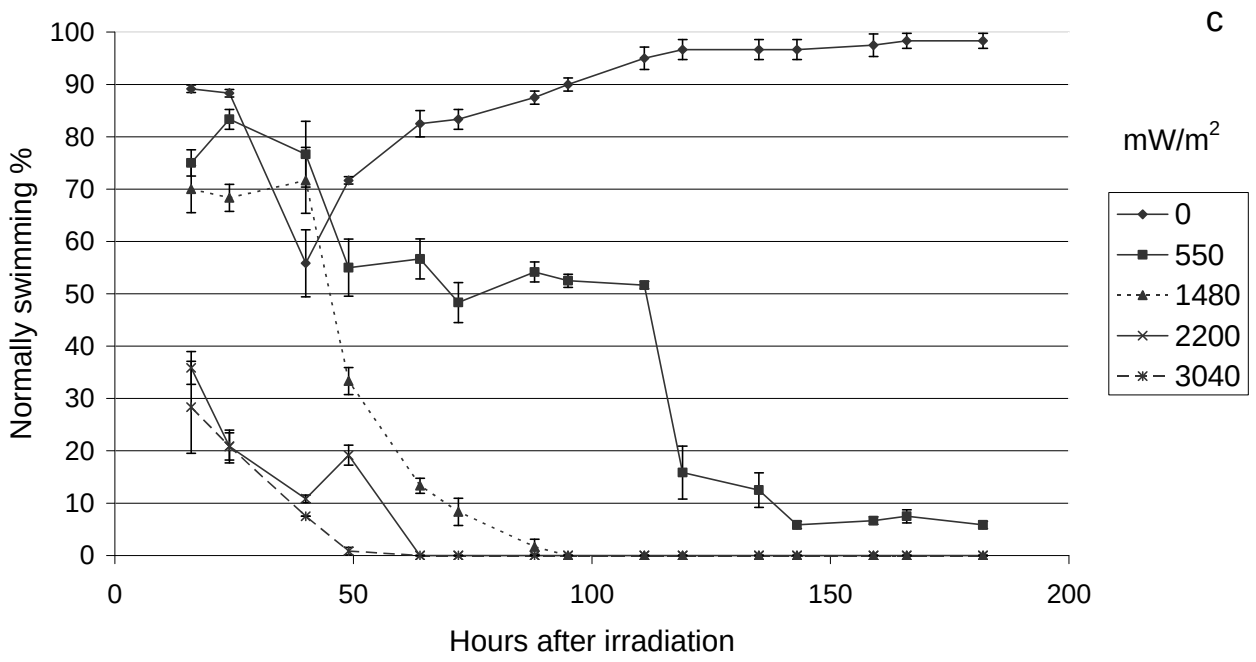
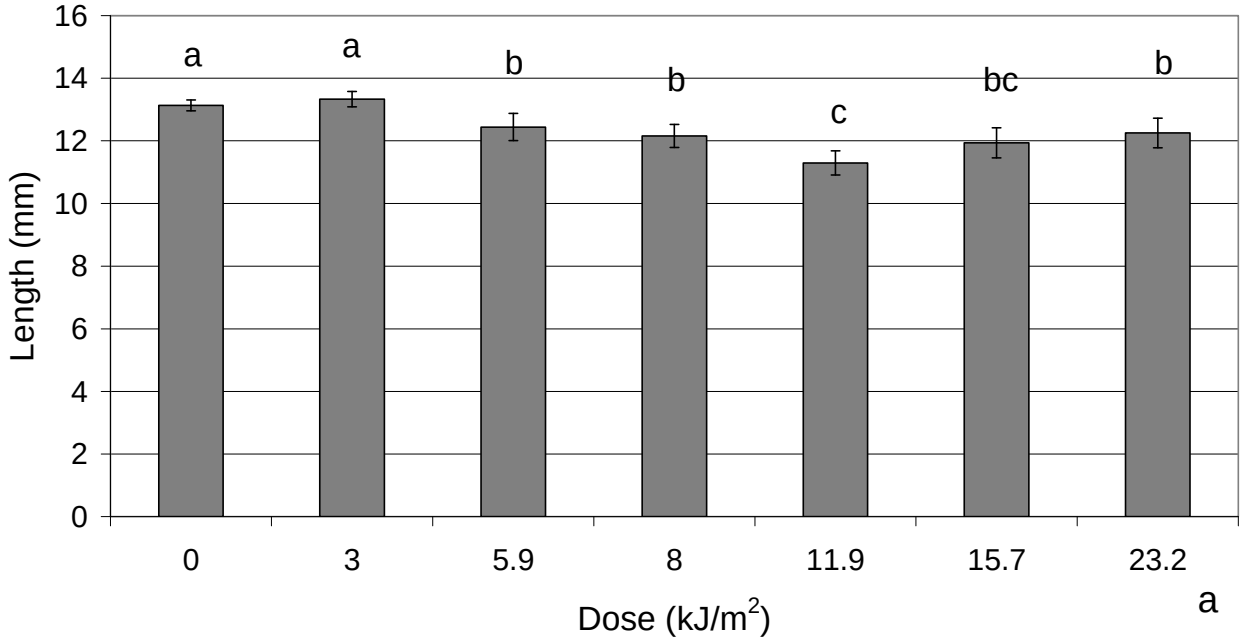

c
Normally swimming %
Hours after irradiation
mW/m2
0
550
1480
2200
3040

Figure 4.


16
14
12
10
8
6
4
2
0
Length (mm)
a
a
b
b
c
bc
b
0
3
5.9
8
11.9
15.7
23.2
Dose (kJ/m2)
a

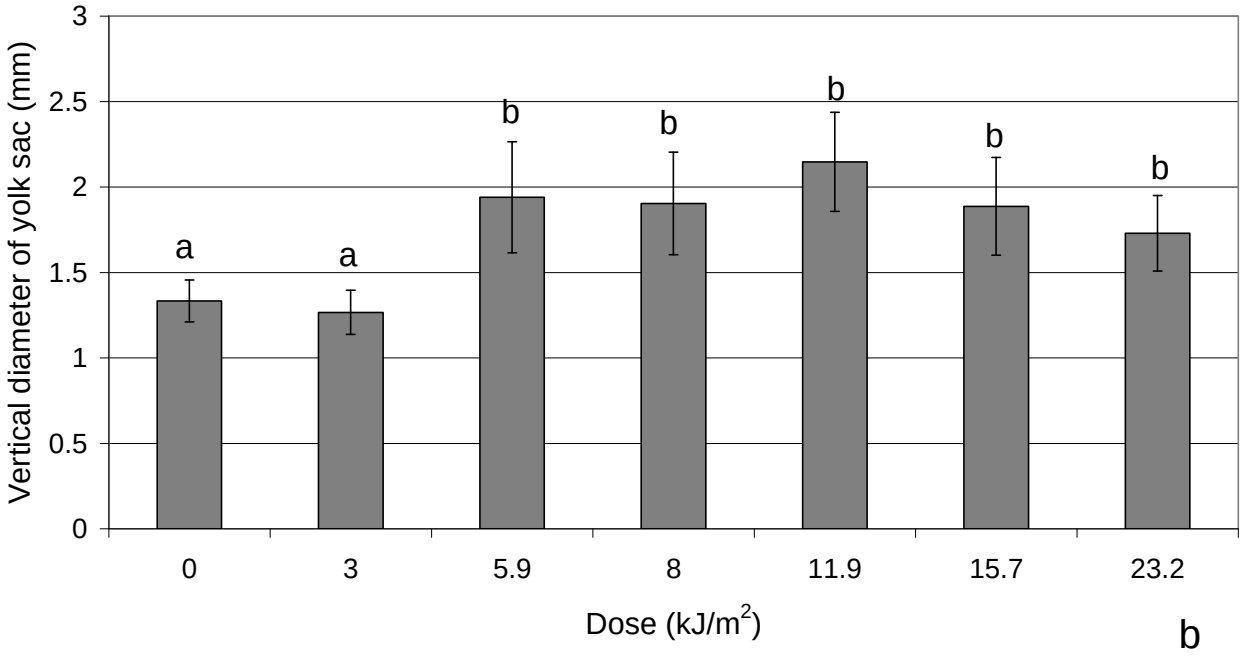

3
2.5
2
1.5
1
0.5
0
Vertical diameter of yolk sac (mm)
a
a
b
b
b
b
b
0
3
5.9
8
11.9
15.7
23.2
Dose (kJ/m2)
b

Figure 5.