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Effects of STP effluents on development and reproduction of the harpacticoid copepod *Nitocra spinipes*

Are matrix population models adequate tools to evaluate data from lifecycle studies?

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1. Introduction

1.1 Background

Pharmaceuticals are a class of emerging environmental contaminants that have been detected at low levels in sewage treatment plant (STP) effluents, surface and seawaters, groundwater and even some drinking waters of many countries (Ternes, 1998; Huggett *et al*., 2003; Fent *et al*., 2006). In STP effluents, various pharmaceuticals usually occur in the ng l^{-1} to µg l^{-1} range, whereas in surface and seawaters they can generally be found in the ng l^{-1} range (Kolpin *et al*., 2002; Kümmerer, 2004; Fent *et al*., 2006). This knowledge was largely facilitated by improvements in environmental residue analysis, i.e. the establishment of methods to detect trace levels of polar organic compounds (Fent *et al*., 2006). Pharmaceuticals enter the aquatic environment through different pathways. Industrial or hospital wastewater and landfill leachates may contain significant concentrations (Holm *et al*., 1995; Kümmerer, 2001). However, after normal application, many of these compounds are eliminated from the body through the renal and/or biliary system and are subsequently disposed via municipal wastewater. If not readily degraded in the STP, these substances are being discharged in treated effluents (Fent *et al*., 2006; Jjemba, 2006). STP effluents are therefore of high priority to environmental risk assessment (ERA) of pharmaceuticals in industrial countries with comprehensive sewage systems. Nevertheless, effects of these effluents containing complex mixtures of different substances on biota are hardly investigated.

In wastewater treatment, two elimination processes are of special importance: biodegradation and sorption to suspended particles (e.g., sewage sludge). Both processes are largely dependent on physical and chemical parameters (e.g., pH, temperature) and thereby determine the removal efficiency. According to the treatment technology, hydraulic retention time and, e.g., the season, removal efficiencies for a single substance can vary strongly between different STPs or samplings at one particular STP. Diclofenac, e.g., a commonly used non-steroidal anti-inflammatory drug (NSAID) was removed to an extent of 0 or 75 %, respectively, during the treatment processes of two different facilities (Andreozzi *et al*., 2003; Tauxe-Wuersch *et al*., 2005). Thus, the regular sewage treatments have to be complemented with additional techniques to ensure reliable removal. Activated carbon or membrane filters, ozone gassing or UV irradiation and parallel addition of oxidizing agents (e.g., hydrogen peroxide) represent possible alternatives. However, performance of these systems and possible other adverse effects to the aquatic environment are largely unknown.

Pharmaceuticals are intended to have a biological effect, which makes them potentially harmful xenobiotics. First, they are relatively persistent to avoid being inactivated before reaching their site of action. Second, they often have similar physio-chemical properties to harmful xenobiotics, e.g. they are able to be transported across membranes. Last, most pharmaceuticals target a specific mode of action (MOA), e.g., COX-inhibitors, β-adrenergic receptor blockers or 17α-Etinyletradiol (Sanderson *et al*., 2004). In ERA, concentrations causing effects in the studied organisms are compared to estimated concentrations of exposure in the environment. Thus, adequate test systems have to be chosen to be able to detect effect concentrations. Moreover, chronic tests and MOA related endpoints are needed for an accurate ERA, especially concerning pharmaceuticals. However, the current literature about ecotoxicological effects deals mainly with acute toxicity measured with traditional standardized tests. This approach only has the ability to detect baseline toxicity of pharmaceuticals (Van Leeuwen *et al*., 1992; Verhaar *et al*., 1992) and is insensitive to specific MOAs, thereby bearing the risk of false negative results in risk assessment (Fent *et al*, 2006). Thus, it is necessary to conduct chronic tests mirroring long-term effects (e.g., lifecycle studies) with representative organisms, e.g., harpacticoid copepods.

1.2 Test organisms

N. spinipes is a benthic bottom-dwelling harpacticoid copepod that is widely distributed around the world (Lang, 1948). Due to its ability to acclimatize to fluctuations of temperature (0-26°C) and salinity (0-30 ‰) (Noodt, 1970; Wulff, 1972) it is suitable for laboratory cultivation and subsequent use in toxicity testing. Bengtsson (1978) described an acute toxicity test using *N. spinipes* which has been established as Swedish and International Standards (ISO). Furthermore, a procedure comparable to the full lifecycle test performed in this study has been described by Breitholtz & Bengtsson (2001).

After accomplishing 6 naupliar stages (NI – NVI) and five copepodite stages (CI – CV), N. spinipes reaches sexual maturity. Nauplii body length range from 90 to 200 µm, copepodites from 230 to 520 µm. The adult female (about 750 µm) is generally bigger that the male (450 – 560 µm). At 20°C, generation time averages 16 – 18 days (Breitholtz & Bengtsson, 2001).

1.3 Stage-structured matrix population models

Life cycle tests provide measures of a large number of endpoints with impact on the population level, e.g., stage-specific survival, developmental rates and reproductive rates (Chandler *et al*., 2004). Generally, multi-generational implications of the obtained data from one lifecycle can be predicted using population models. Among others (e.g., individual based models, differential equation models), stage-structured Lefkovich population matrices can be used for this purpose. In this modification of Leslie matrix population models (Leslie, 1945), mortality, transition rates and fecundity are not determined by the age but by the developmental stage of an individual (Lefkovich, 1965). Hence, this method is appropriate if the lifecycle of the test organism, e.g., *N. spinipes*, can be divided into discrete stages (e.g., nauplii, copepodites, adults). In short-term projections of those matrices, dynamics of a population are represented until a stable age distribution is compassed. Subsequently, projections equal a simple exponential growth with a discrete finite rate of increase, λ (Caswell, 2001). Furthermore, confidence intervals (CIs) and extinction risks for the obtained matrix projections can be calculated using Monte Carlo methods that implement the variation of the empiric data measured in the lifecycle test (Tenhumberg *et al.*, 2008).

1.4 Aim of the study

The aim of this study was to investigate the effects of effluents from the STP Henriksdal, Stockholm, Sweden, on growth and development of the harpacticoid copepod *N. spinipes* using a full lifecycle test system. Different additional treatment techniques were applied to the native sand-filtered effluent in a medium scale pilot process at the STP to evaluate an appropriate method to remove pharmaceuticals or other organic chemicals (e.g., personal care products). The two methods used for this study were (I) an activated carbon filter and (II) UV irradiation with parallel addition of hydrogen peroxide. The second aim of this study was to prove the suitability of stage-structured matrix population models to represent effects on the population level as an integration of a variety of measured endpoints during full lifecycle tests. Particularly, this method could alleviate the interpretation of these tests and be beneficial to future legislative.

2. Materials and Methods

2.1 Test organisms

The harpacticoid copepod *Nitocra spinipes* Boeck used in the present study was isolated from a sediment sample in the Tvären Bay, Baltic Sea, in 1975. Detailed information on the used strain and culturing conditions has been published elsewhere (e.g., Bengtsson, 1978; Breitholtz & Bengtsson, 2001). In brief, permanent stock cultures are maintained in darkness at 22 ± 1°C in approx. 100 ml GF/C-filtered (Schleicher & Schuell, Dassel, Germany) and pre-heated (80°C) natural brackish water (salinity 6 - 7 ‰, water used throughout the whole test) and fed weekly with commercial salmon feed (Astra-Ewos, Södertälje, Sweden).

2.2 STP effluents and experimental design

Three different treatment techniques (I-III) were investigated in a pilot process at the STP Henriksdal, Stockholm (Fig. 1). Effluent samples from these processes were taken from March 13 to March 16 2008 and used in the present lifecycle study. Having passed the different sewage treatment processes, particulate matter and sorbed contaminants were removed from the influent water by a sand filter (I). This water was further cleaned up using (II) an active carbon-filter or (III) ultraviolet radiation (UV) and hydrogen peroxide (H_2O_2). Active carbon is extremely porous and thus has a large surface area available for adsorption of a variety of compounds and contaminants. Hydrogen peroxide is a strong oxidizing agent and could have (in combination with energy addition by UV irradiation) the ability to degrade organic compounds. All sewage effluent samples were stored at -20°C until use. Samples were thawed carefully in darkness using 15°C water prior to the experiment and each water renewal. The salinity of all samples was adjusted to that of the GF/C-filtered brackish water used in the control treatment with sodium chloride (analytical grade, Merck Darmstadt). Dissolved oxygen and pH were monitored to ensure appropriate culturing conditions. Subsequently, each solution was diluted with GF/C filtered natural brackish water to three concentrations: 3, 15 and 75 %. Thereafter, these dilutions were used in the full lifecycle test (see *lifecycle test*), where two microplates (i.e., 72 animals) were exposed to each concentration.

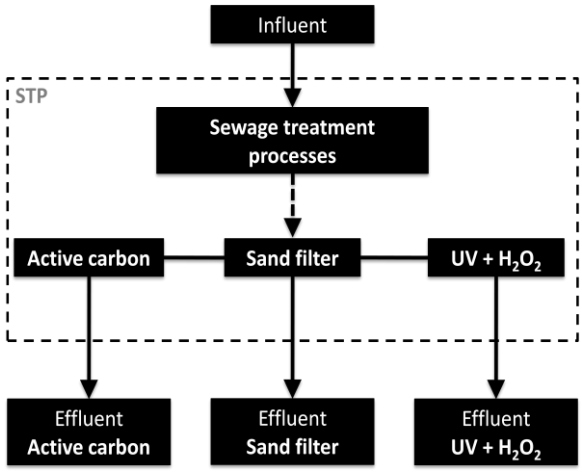

Figure 1. Schematic flow chart of the pilot process installed at the STP Henriksdal. The three different effluents (sand-filtered, active carbon- and UV/H_2O_2-treated effluents) were investigated in this study.

2.3 Algal cultures

A mono-diet with the alga *Rhodomonas salina* (Cryptophyceae) has been shown to permit the best developmental and reproductive performance in lifecycle studies with *N. spinipes* (Dahl *et al*., 2009) and was therefore chosen for the present study. Algae were grown in 10 ‰ f/2 medium (autoclaved 0.2 µm filtered natural seawater, with 75 mg l^{-1} KNO_3, 5 mg l^{-1} NaH_2PO_4 and a mixture of vitamins and trace minerals, at pH 8.0) and maintained at room temperature (22 ± 1°C) with rear illumination at approx. 20 µE m^{-2} s^{-1}. After approx. 7 days, the cultures were transferred to autoclaved 1 l screw cap glass bottles (Schott Duran®) and allowed to settle in the refrigerator (2 – 8°C). Supernatant medium was removed using a suction pump and the remaining algal suspension was transferred to an autoclaved 100 ml screw cap glass bottle (Schott Duran®). The algal concentration was measured using an electronic particle counter (Beckman Coulter, Z2 Particle Count and Size Analyzer). Suspensions were stored in the refrigerator (2 – 8°C) until usage for a maximum of 4 days until they were used.

2.4 Lifecycle test

The lifecycle test in the present study followed methods described elsewhere (e.g., Dahl *et al*., 2009). Briefly, females with well-developed egg sacs were isolated from the permanent culture. Nauplii released within 24 hours (stage NI) were collected and randomly allocated into individual wells (36 individuals per plate, wells B2 – G7) of medium binding 96-well polystyrene microplates with lids (Corning Costar EIA/RIA). STP effluents were prepared (see *STP effluents*) and *R. salina* suspension was added to a final concentration of $5.0 \cdot 10^7$ cells ml^{-1} to both effluents and GF/C-filtered natural brackish water in the control treatment, respectively. Subsequently, the suspensions were added to a final volume of 270 µl at the desired concentration to each well. Microplates were soaked for 24 h in deionized water prior to the experiment. All unused wells were filled with 270 µl GF/C-filtered natural brackish water to maintain constant humidity throughout all used wells. The plates were kept in darkness at 22 ± 1°C. Test solutions were renewed (70 %) three times a week and copepods fed simultaneously. Dissolved oxygen, salinity and pH of the removed water were controlled at each renewal. Individuals were monitored daily using a stereo-microscope and survival and developmental progress were recorded.

On mating day (i.e., day 23 of the test), the majority of individuals had developed to mature adults and was collected by sex within each treatment. Animals were mated pairwise in individual wells of 24-well polystyrene microplates with lids (Corning Costar EIA/RIA) containing 2 ml of sample dilutions as described above. Test solutions were renewed (70 %) three times a week and copepods fed simultaneously. Dissolved oxygen, salinity and pH of the removed water were controlled at each renewal. Microplates were soaked for 24 h in deionized water prior to the experiment. Mating pairs were monitored daily using a stereo-microscope. Survival, reproductive success (females producing at least two viable clutches) and brood sizes were recorded.

2.5 Stage-structured matrix population modelling

Multi-generational effects on the population level were modelled using Monte Carlo simulations of stage-structured Lefkovich matrices (Caswell, 2001; Lefkovich, 1965; Leslie, 1945). The following four-stage (nauplius to copepodite to female to ovigerous female) matriarchal model (*Formula 1*) was

applied to project population dynamics through four generations and to calculate the finite rate of increase (λ; Caswell, 2001).

$$\begin{pmatrix} N_n \\ N_c \\ N_f \\ N_{fo} \end{pmatrix}_t = \begin{pmatrix} P_{nn} & 0 & 0 & F \\ P_{nc} & P_{cc} & 0 & 0 \\ 0 & P_{cf} & P_{ff} & 0 \\ 0 & 0 & P_{ffo} & 0 \end{pmatrix} \times \begin{pmatrix} N_n \\ N_c \\ N_f \\ N_{fo} \end{pmatrix}_{t-1}$$

Formula 1. $\boldsymbol{N}$ represents the number of individuals in a certain stage class and $\boldsymbol{P}$ the life stage transition rate. Indices represent the different stage class: $\boldsymbol{n}$ – nauplius, $\boldsymbol{c}$ – copepodite, $\boldsymbol{f}$ – female, $\boldsymbol{f_O}$ – ovigerous female (i.e., female producing at least two viable clutches).

The proportions used in the matrix were based on ($\boldsymbol{P_{nn}}$) the proportion of nauplii not developing to copepodites, ($\boldsymbol{P_{nc}}$) nauplii developing to copepodites, ($\boldsymbol{P_{cc}}$) copepodites not developing to females, ($\boldsymbol{P_{cf}}$) copepodites developing to females and thereby capturing sex ratio shifts, ($\boldsymbol{P_{ff}}$) females not producing at least two viable clutches, ($\boldsymbol{P_{ffo}}$) females producing at least two viable clutches and ($\boldsymbol{F}$) the average number of offspring per female. Stage-specific mortality was included in each step. Since this model is matriarchal, the resulting population abundance projections should be considered as comparisons relative to the control treatment, not as absolute field abundances.

Monte Carlo simulations were performed by generating matrices with random proportions and fecundities from the distributions defined by the test data (Manly, 1991). Proportions were assumed to be binomially distributed, while fecundity was assumed to be normally distributed. Overall, 10000 random matrices were generated and the expected short-term population dynamics of an initial F_0 cohort of 72 animals (i.e., the number of animals in each treatment) were projected through four generations. Mean values and 95% confidence Intervals (CIs) were calculated automatically. Concerning the Monte Carlo simulations of λ, the same procedure was computed though 1000 iterations with subsequent calculation of λ instead of matrix projections. Mean values and 95% confidence Intervals (CIs) were calculated automatically.

All matrix and Monte Carlo simulations were performed using the free Microsoft Excel™ Plug-in Pop Tools 3.0 (http://www.cse.csiro.au/poptools, build 6, released 2008-09-01) and Microsoft Excel™ 2007.

2.6 Statistical analysis and graphical presentation

All spreadsheet calculations were performed using Microsoft Excel™ 2007. All graphs were plotted using the program Graph Pad Prism 5. Statistical analyses and comparisons were conducted using the software Sigma Stat 3.11. Treatment proportions (e.g., survival) were statistically compared with the control treatment using a *z*-test with Yates correction. Average times of development and fecundity were compared to the control treatment using non-parametric Kreskas Wallis one way ANOVA on ranks and Dunn's method, since the test data did not pass a Kolmogorov-Smirnov normality test ($p \leq 0.001$). Developmental dynamics were compared using a Cox regression (Breslow method for ties). Statistical significance limit throughout all comparisons was set at $p \leq 0.05$. If not stated differently, all errors are expressed as standard errors of the mean (SEM).

3. Results

3.1 Survival success

Survival in the control treatment observed throughout the whole test remained high (91.1 %). The test was therefore considered as valid (threshold value: 20% mortality). The three different treatments showed a dose-dependent pattern, where the higher concentrations caused higher mortality (*Figure 2*). While the UV/H_2O_2-treated effluent showed no significantly reduced survival compared to the control group (z-test with Yates correction, $p \leq 0.05$) throughout all concentrations, survival at the highest concentration (75%) of the sand-filtered effluent was significantly reduced by 18.6 % ($p \leq 0.05$). The lowest survival throughout all treatments was observed for the 75% dilution of the active-carbon treated effluent (58.3 %; significant, $p \leq 0.05$). Furthermore, this effluent was the only treatment causing a significant reduction of survival at a medium concentration (15 %). For further details on the change of survival with time, see *Figure 3, A-C*.

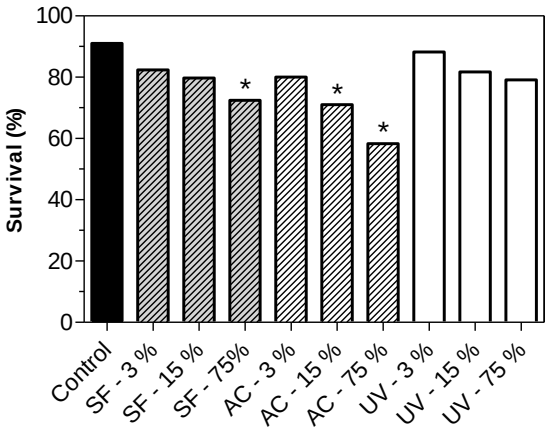

Figure 2. Summarized survival (%) throughout the whole test. **SF**: sand-filtered effluent, **AC**: active carbon-treated effluent, **UV**: UV/H_2O_2-treated effluent. *Significantly lower survival compared to the control treatment (z-test with Yates correction, $p \leq 0.05$, $n = 72$).

3.2 Developmental success

Developmental success was observed from the naupliar stage to copepodites (NI to CI developmental success) and from copepodites to adults (CI to A developmental success). Both stage transitions were described by the average day of development to the final stage (*Figure 5 and 7*). Temporal developmental dynamics were described by plotting the percentage of individuals in the final stage at each day of the test (*Figure 4, A-D and 6, A-D*).

First, the overall developmental success (i.e., the final percentage of individuals developing to the final stage) equalled 100 % for both transitions in the control group and was not significantly reduced in any other treatment. The lowest percentage of nauplii developing to copepodites was observed at the 75 % dilution of the sand-filtered effluent (80.6 %, not significant; *Figure 4, B*), while the lowest

percentage of copepodites developing to adults was observed at the 75 % dilution of the active carbon-filtered effluent (93.5 %, not significant; *Figure 6, C*).

Second, the average day of development to copepodites and adults was calculated. While the individuals in the control group developed to copepodites after 7.2 ± 0.1 days and to adults after 14.1 ± 0.2 days, the average day of development was not significantly altered in any other treatment (*Figure 5 and 7*).

Concerning the temporal dynamics of both transitions, development to copepodites was not altered significantly by any treatment (Log Rank comparison, $p = 0.21$; *Figure 4, A-D*). Development to adults instead departed from the developing of values from the control treatment in the different sand filter (all concentrations; Cox regression, $p \leq 0.05$) and the active carbon treatments (medium and high concentration; Cox regression, $p \leq 0.05$; *Figure 6, A-D*). *Table 1* presents the results from the Cox regression (i.e., *p*-values).

Table 1 – Alteration of the development from copepodites to adults as represented by the *p*-values from a Cox regression. Values lower than 0.05 indicate that development to adults departed from the developing of values from the control treatment. Numbers in italics indicate significantly altered development.

Concentration	**Sand-filtered**	**Active carbon**	**UV/H_2O_2**
Low (3 %)	*0.029*	0.125	0.085
Medium (15 %)	*0.011*	*0.040*	0.183
High (75 %)	*0.004*	*0.000*	0.070

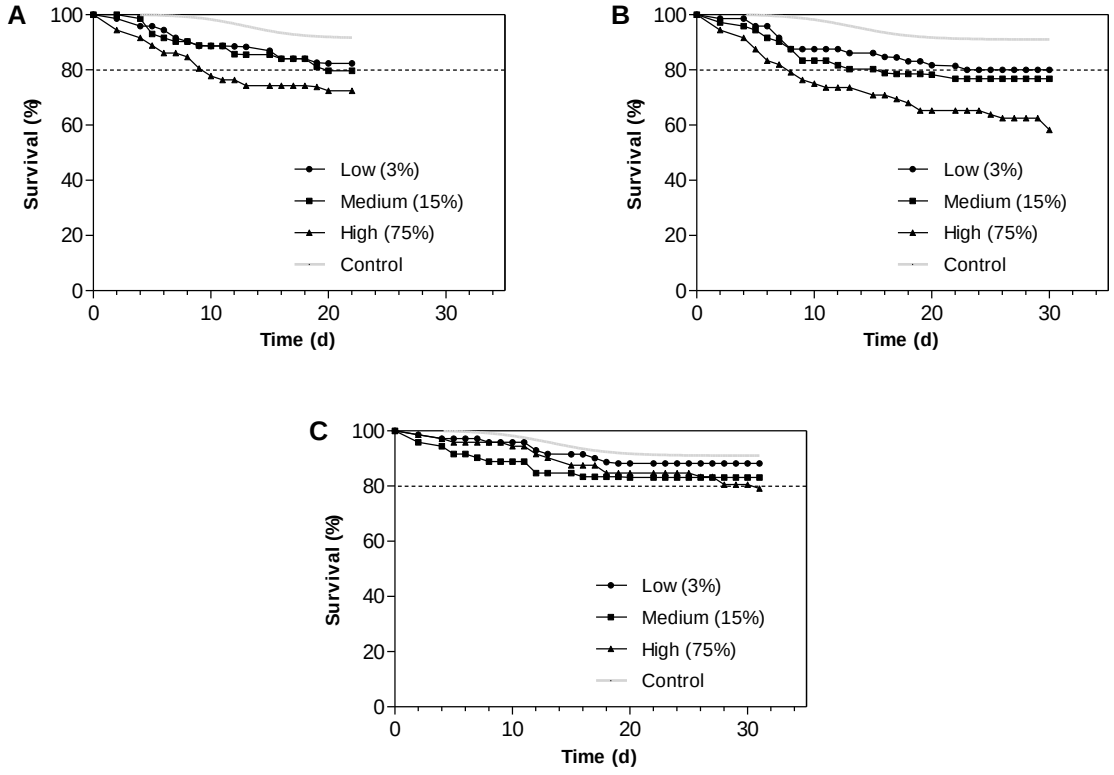

Figure 3. Survival success (%) at each day observed throughout the whole test (until mating). **A**: sand-filtered effluent, **B**: active carbon-treated effluent, **C**: UV/H_2O_2-treated effluent. The grey line represents the control treatment; black lines represent the different treatment levels.

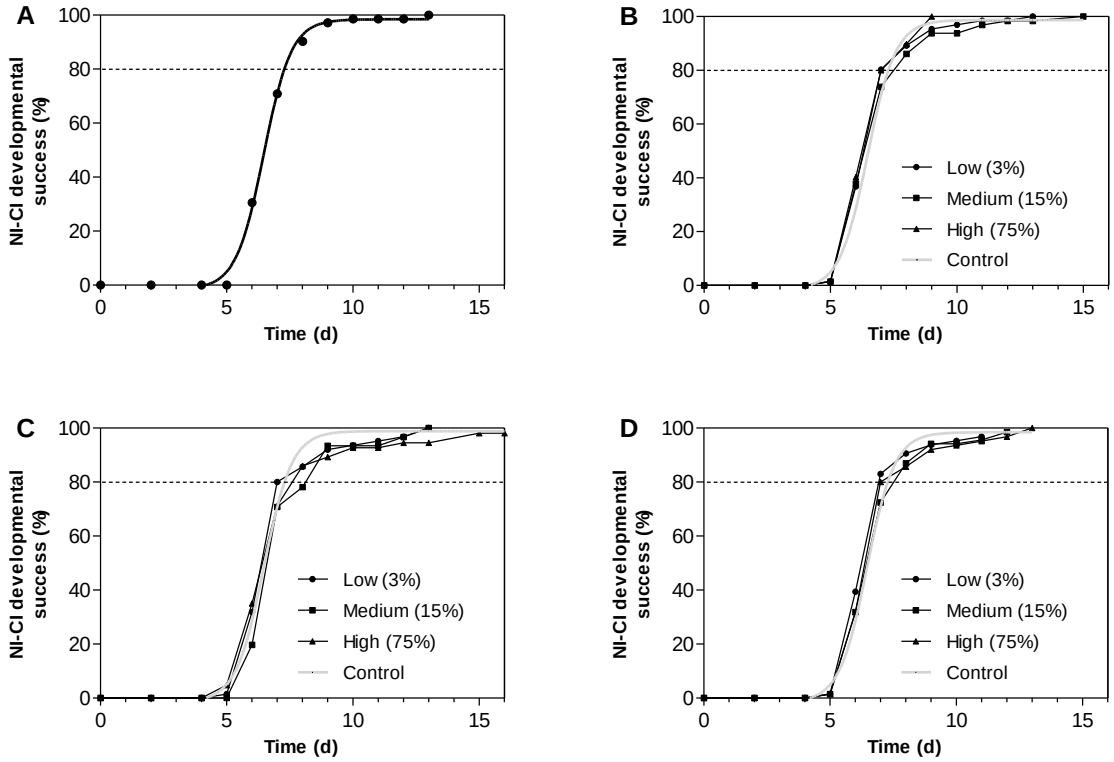

Figure 4. Nauplius to copepodite (NI-CI) developmental success (%) throughout the whole test among surviving animals. **A**: control treatment, **B**: sand-filtered effluent, **C**: active carbon-treated effluent, **D**: UV/H_2O_2-treated effluent. The grey line represents a sigmoid regression with variable slope of the control treatment data; black lines represent the different treatment levels.

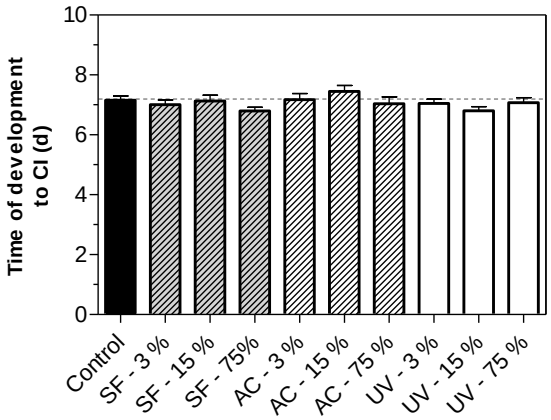

Figure 5. Average day of development to copepodites (CI) **SF**: sand-filtered effluent, **AC**: active carbon-treated effluent, **UV**: UV/H_2O_2-treated effluent. The dashed line represents the average day of development to CI in the control treatment.

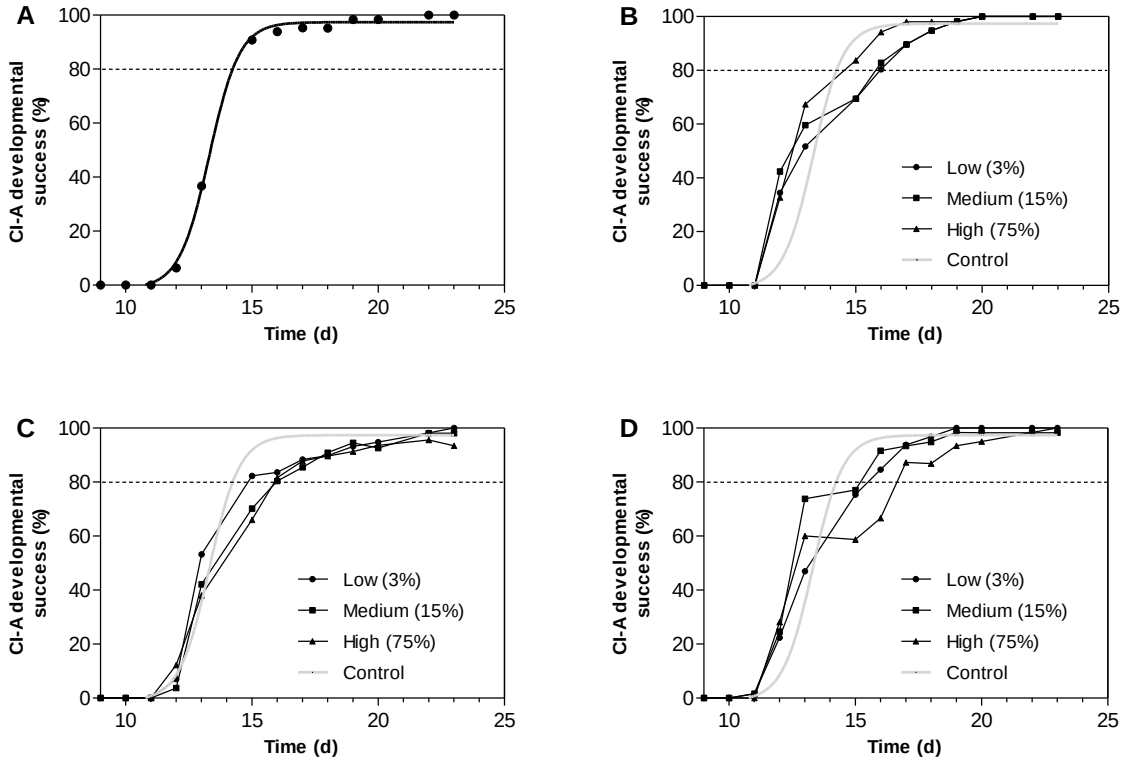

Figure 6. Copepodite to adult (CI-A) developmental success (%) throughout the whole test among surviving animals. **A**: control treatment, **B**: sand-filtered effluent, **C**: active carbon-treated effluent, **D**: UV/H_2O_2-treated effluent. The grey line represents a sigmoid regression with variable slope of the control treatment data, the black lines the different treatment levels.

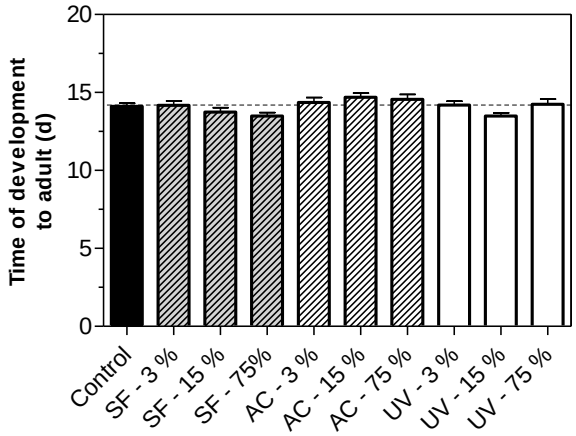

Figure 7. Average day of development to adults (A) **SF**: sand-filtered effluent, **AC**: active carbon-treated effluent, **UV**: UV/H_2O_2-treated effluent. The dashed line represents the average day of development to adults in the control treatment.

3.3 Sex-ratio and fecundity

The final sex ratio (i.e., the number of male individuals divided by the number of female individuals) in the control treatments equalled one, while some of the treatments caused altered sex ratios. Generally, the medium concentrations caused the highest effects. At the 15 % dilution of the sand-filtered effluent, e.g., 3-fold more males than females were observed (*Figure 8*).
Females in the control treatments produced an average offspring of 36.3 ± 12.8 nauplii (*Figure 9*). Throughout the other treatments, trends indicate a decreased number of nauplii at higher concentrations. However, changes are not significant.

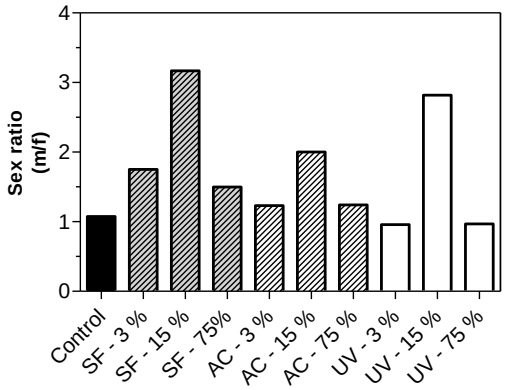

Figure 8. Final sex ratio (number of males divided by number of females. **SF**: sand-filtered effluent, **AC**: active carbon-treated effluent, **UV**: UV/H_2O_2-treated effluent.

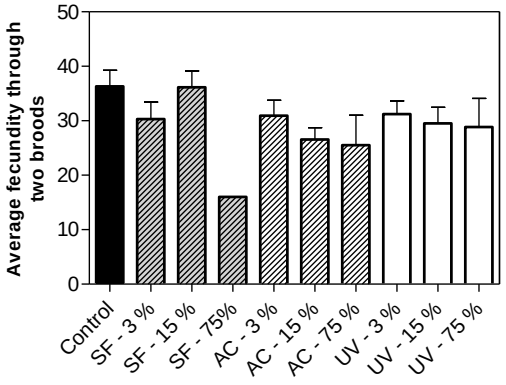

Figure 9. Average fecundity through two broods. Bars represent mean ± SEM. **SF**: sand-filtered effluent, **AC**: active carbon-treated effluent, **UV**: UV/H_2O_2-treated effluent.

3.4 Stage-structured matrix population modelling

Results of the stochastic Monte Carlo Lefkovich matrix model were expressed as the finite rate of increase (λ) and as abundance projections throughout four generations (i.e., 16 time steps). Abundance projections (*Figure 11, A-J*) represent the short-term behaviour of an initial F_0 cohort of 72 (i.e., the initial number of nauplii used in the test) nauplii throughout the following four generations. *Table 2* shows the mean proportional life stage transitions, which were used to construct the matrices.

Table 2 – Mean proportional life stage transitions (± SD) and fecundities observed during the full lifecycle exposure of N. spinipes exposed to different treatments. **See formula 1 for explanation of the stage transition proportions.*

Treatment	Pnn	Pnc	Pcc	Pcf	Pff	Pfg	F
SF (3 %)	0.00 ± 0.00	0.90 ± 0.03	0.00 ± 0.00	0.42 ± 0.06	0.26 ± 0.08	0.63 ± 0.09	30.31 ± 12.50
SF (15 %)	0.00 ± 0.00	0.88 ± 0.04	0.00 ± 0.00	0.32 ± 0.06	0.18 ± 0.08	0.73 ± 0.09	36.13 ±11.61
SF (75 %)	0.00 ± 0.00	0.81 ± 0.05	0.00 ± 0.00	0.21 ± 0.05	0.45 ± 0.15	0.09 ± 0.09	16.00 ± 00.00
UV (3 %)	0.00 ± 0.00	0.96 ± 0.02	0.00 ± 0.00	0.35 ± 0.06	0.29 ± 0.09	0.71 ± 0.09	31.24 ± 09.95
UV (15 %)	0.03 ± 0.02	0.86 ± 0.04	0.00 ± 0.00	0.42 ± 0.06	0.44 ± 0.10	0.36 ± 0.10	29.54 ± 10.66
UV (75 %)	0.01 ±0.01	0.94 ± 0.03	0.00 ± 0.00	0.28 ± 0.05	0.32 ± 0.11	0.36 ± 0.10	28.86 ± 13.89
AC (3 %)	0.00 ± 0.00	0.88 ± 0.04	0.00 ± 0.00	0.40 ± 0.06	0.35 ± 0.09	0.58 ± 0.10	30.93 ± 11.00
AC (15 %)	0.00 ± 0.00	0.85 ± 0.40	0.00 ± 0.00	0.41 ± 0.06	0.22 ± 0.09	0.78 ± 0.09	26.56 ± 09.22
AC (75 %)	0.01 ± 0.01	0.74 ± 0.05	0.00 ± 0.00	0.21 ± 0.06	0.58 ± 0.14	0.33 ± 0.14	25.50 ± 11.00
Control	0.00 ± 0.00	1.00 ± 0.00	0.00 ± 0.00	0.43 ± 0.06	0.30 ± 0.08	0.67 ± 0.09	36.32 ± 12.88

While most modelled populations grew constantly throughout all treatments and levels (as represented by the mean of 10,000 Monte Carlo simulations) population growth in the 75 % dilution of the sand-filtered effluent (*Figure 11, D*) was low from the start (and remained that way). Regarding the stochastic information from this kind of model, the lower 95 % confidence interval intersected the x-axis at the high concentration of all effluent treatments (*Figure 11; D, G, J*) , expressing that a certain risk of extinction is given within a probability of 95 %.

This risk was quantified by calculating the percentage of finite rates of increase where $\lambda < 1$ throughout 1,000 Monte Carlo iterations, giving the probability of populations occurring in this test system where population growth was negative (*Table 2*). While the extinction risk was relatively low throughout all treatments at the 3 and 15 % dilutions (0.1 – 2.4 %) compared to the control treatment (0.3 %), the higher concentrations caused elevated risks. The risk was highest exposed to the sand-filtered effluent, where the population was extinct with a probability of 84.9 % (*Table 3*).

Corresponding to the extinction risks, the same data was used to plot the average finite rate of increase (λ) as the mean value throughout 1,000 Monte Carlo iterations. The higher concentrations in general showed lowered rates of increase. Correspondingly, the sand-filtered effluent was the only effluent which caused an average population growth rate lower than 1 (i.e., the population diminished at a rate of 0.74).

Table 3 – Extinction risk of modelled populations exposed to the different treatments expressed as the percentage of finite rates of increase where $\lambda < 1$ throughout 1,000 Monte Carlo iterations.

Concentration	**Extinction risk (%)**			
	Control	**Sand-filtered**	**Active carbon**	**UV/H_2O_2**
Low (3 %)	0.3	2.4	0.7	0.5
Medium (15 %)		0.1	0.9	0.9
High (75 %)		84.9	14.4	8.2

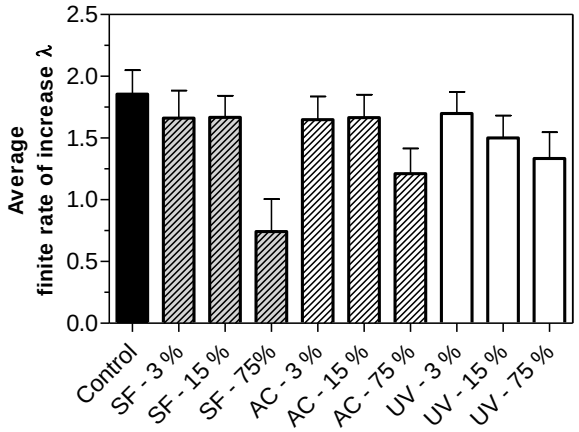

Figure 10. Finite rate of increase (λ) calculated from stochastic Lefkovich matrices. Bars represent the mean ± 1 SD of 1,000 Lefkovich matrices generated with the Monte Carlo method. **SF**: sand-filtered effluent, **AC**: active carbon-treated effluent, **UV**: UV/H_2O_2-treated effluent.

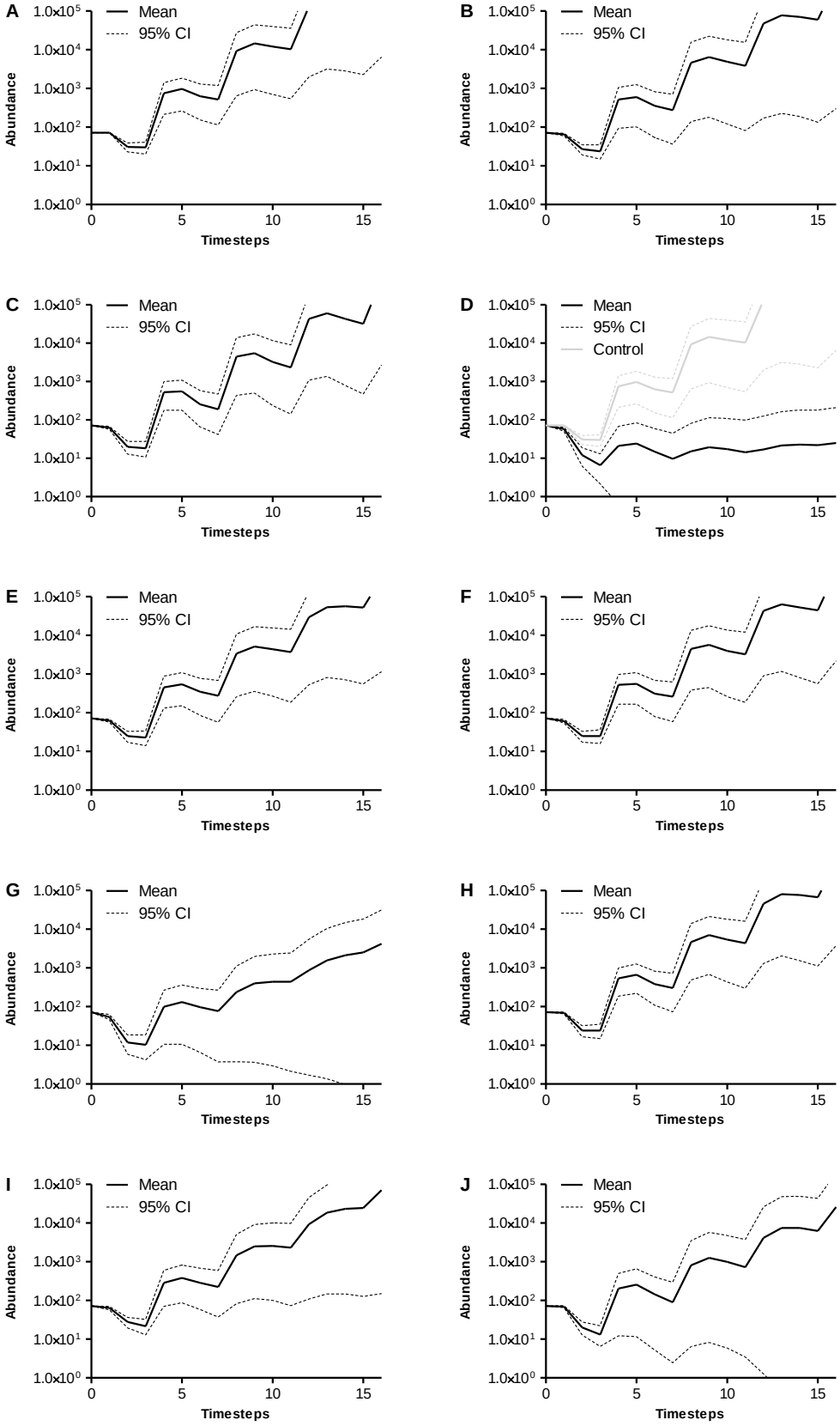

Figure 11. Mean values and 95% confidence intervals of 10,000 Monte Carlo Lefkovich matrix projections of an F_0 cohort of 72 nauplii throughout 4 generations (16 time steps). **A**: control, **B-D**: sand-filtered effluent at 3, 15 and 75 %, respectively. **E-G**: active carbon-filtered effluent at 3, 15 and 75 %, respectively. **H-J**: UV/H_2O_2 treated effluent at 3, 15 and 75 %, respectively.

4. Discussion

4.1 Full lifecycle study

The major aim of this study was to investigate the effects of effluents from the STP Henriksdal, Stockholm, Sweden, on growth and development of the harpacticoid copepod *N. spinipes* using a full lifecycle test system. Different additional treatment techniques were applied to the native sand-filtered effluent in a medium scale pilot process at the STP to evaluate an appropriate method to remove pharmaceuticals or other organic chemicals (e.g., personal care products). The two methods used for this study were (I) an activated carbon filter and (II) UV irradiation with parallel addition of hydrogen peroxide. Both treated effluents and the native sand-filtered effluent, respectively, were investigated in a full lifecycle test system using the harpacticoid copepod *N. spinipes*. Both additional treatments showed to improve the quality of the STP effluents, while the active carbon-filtration seemed to be the preferable method that caused the lowest effects on population-relevant endpoints. However, the cost-benefit ratio should be regarded critically.

In environmental risk assessment (ERA), especially of pharmaceuticals, many toxicity tests focus on easily observable endpoints at the individual level (e.g., mortality, growth inhibition) for economical or logistical reasons. However, alterations at this level are not necessarily correlated with responses at higher levels of biological organization (Gardeström *et al.*, 2008; Fent *et al*., 2006; Forbes & Calow, 1999). Many aquatic species are continuously exposed to pharmaceuticals over long periods or even over their whole lifecycle, e.g., in recipients of STP effluents (Fent *et al*., 2006). The full lifecycle test system used in this study is therefore an appropriate method to cover all possible alterations of survival, growth, development and reproduction for all different life stages of *N. spinipes* and has the potential to provide useful information to risk assessment, especially concerning the highly complex mixture effects. Nevertheless, this experimental setup should be chosen carefully for the high economic expenditure. In some cases, partial lifecycle tests could be of comparable informative value.

When comparing the different investigated treatments, both additional techniques (active carbon-filtered effluent, UV irradiated and hydrogen peroxide treated effluent) generally caused lower effects compared to the control treatment than the native sand-filtered effluent; except for the mortality in the active-carbon treated effluent. Additionally, this was reflected by chemical analyses that were conducted in a parallel study with the same effluents, where both treated effluents contained lower concentrations of the analyzed pharmaceuticals (Metoprolol, Oxazepam, Propranolol, Ranitidin, Terbutalin, Carbamazepine, Cyclophosphamide, Paracetamol, Ketoprofen, Diclofenac, Furosemide, Gemfibrozil, Hydrochlorthiazide, Ibuprofen, and Naproxen). The active carbon filtration was the more effective removal method (T. Alsberg, personal communication, December 2008).

Mortality over all was moderate despite of the long-term exposure, not exceeding 50 % in any treatment, and showed a concentration-dependent pattern (*Figure 3, A-C*). Lethal effects were highest in the active carbon-filtered effluent even though pharmaceutical concentrations were lowest in this treatment. However, levels of total organic carbon (TOC) were lowered by approximately 75 % by this technique (B. Björlenius, personal communication, December 2008) and a high change of mortality occurred during the first week (i.e., naupliar stage). A possible explanation of the high mortality could be a hypothetical interference between the effects that were caused by the pharmaceuticals in the effluent and effects caused by a deficiency of accessible nutrients (i.e., SPM that nauplii feed on). Future test designs should be modified to consider SPM as a potential source of data variation.

The average day of development from nauplii to copepodites and from copepodites to adults, respectively, was not affected by any treatment. It was neither delayed, nor accelerated. The temporal dynamics of development to copepodites was not altered significantly by any treatment (*Figure 4, A-D*). The transitions to adults instead departed from the control treatment in the sand filter and active carbon treatments (*Figure 6, A-D*) as indicated by a Log Rank comparison. A small number of animals

in the sand-filtered effluent merged to the adult stage earlier, while this took marginally longer for some animals exposed to the active carbon-filtered effluent. Although, an impact on the population level of a small group showing impaired development is unlikely.

The final sex ratio (i.e., the number of male individuals divided by the number of female individuals) in the control treatments equalled one, while some of the treatments caused altered sex ratios. Generally, the medium concentrations caused the highest effects (*Figure 8*). Possibly, this pattern was caused by selective mortality at medium concentrations (i.e., more females were affected by the pharmaceuticals than males), while the decreased number of males at high concentrations could have been caused by endocrine disruption. The reproductive output per female (i.e., the fecundity) seemed not to be influenced by the treatments (*Figure 9*). However, only one female produced two viable clutches at, e.g., the high concentration of the sand-filtered effluent. That high variation between the sample sizes and the samples apart cause an elevated risk for false negative statistical comparisons using analysis of variance (ANOVA).

4.2 Stage-structured matrix population models

The second aim of this study was to prove the suitability of stage-structured matrix population models to represent effects on the population level as an integration of a variety of measured endpoints during full lifecycle tests. Particularly, these analyses are easily realizable with free software (Pop Tools) and all matrix parameters can be calculated from the empirical data that was obtained from full lifecycle observations without requiring the estimation of any parameter. One central assumption of this approach is the equality of effects throughout all generations, which is defensible since no additional data is available. A major disadvantage of matrix models is the equality of time steps (Lewis, 1945; Lefkovich, 1965; Caswell, 2001). Alterations in developmental times are unattended. Since the average day of development to copepodites and adults, respectively, was not impaired, this disadvantage does not apply to this study. Furthermore, the inclusion of variation from the empiric data by application of Monte Carlo methods to obtain a range of possible effects augments the informative value of matrix projections.

When comparing the different investigated treatments, both additional treatment techniques caused lower effects on the population level than the native sand-filtered effluent, while any treatment caused a lowered finite rate of increase compared to the control (*Figure 10*). Surprisingly, the UV and hydrogen peroxide treated effluent caused a higher effect at a medium concentration than both other treatments (active carbon and sand filtration, respectively). This could be a possible indication of an adverse effect that is caused by the treatment itself. Possibly, oxidation products of the original contaminants could have higher toxic potency than the original compound itself. In summary, the effects as described by this method (finite rate of increase) correlate better with the chemical analysis than the other effects individually, indicating a significant facilitation of the interpretation of lifecycle studies. Especially within environmental legislation, simple indices are required to allow non-experts competent decision making.

However, even the native sand-filtered effluent showed virtually no effect at a dilution of 15 % on the population level. STP effluents will possibly be diluted quickly after discharge to no-effect-concentrations. Regarding the high expenses of the additional treatment techniques at a large scale, the cost-benefit ratio should be regarded critically.

5. Conclusions

First, the two additional treatment techniques used in the pilot process at the STP Henriksdal have the potency to improve the quality of the native sand-filtered effluent. Results from the full lifecycle test conducted within this study showed the active carbon-filtration to be the preferable method that caused the lowest effects on population-relevant endpoints. Second, stage-structured matrix population models are appropriate tools to facilitate the interpretation of full lifecycle data. Especially within environmental legislation, simple indices are required to allow non-experts competent decision making.

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