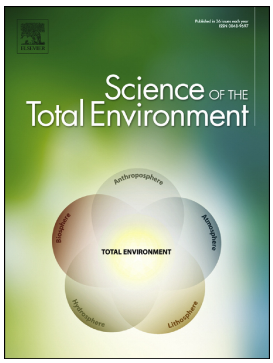

Assessing the acute and chronic toxicity of exposure to naturally occurring oil sands deposits to aquatic organisms using Daphnia magna

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Assessing the acute and chronic toxicity of exposure to naturally occurring oil sands deposits to aquatic organisms using *Daphnia magna*.

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Abstract

In the Athabasca region, the oil sands are located at or near the surface making open-pit mining viable. In addition, the Athabasca River and its tributaries flow through these oil sands deposits, thereby receiving bitumen-associated contaminants through natural fluvial erosional and weathering processes. A key knowledge gap has been related to understanding both the magnitude and significance of the toxicological and ecological effects on aquatic organisms exposed to naturally occurring bitumen entering fluvial systems. Using the Daphnia magna model system, this study assessed the ecotoxicological effects of exposure to bitumen-elutriate treatments that simulated the early stages of fluvial/erosional exposure conditions. No significant among-site differences were observed in the survival of D. magna after 48 h exposure to elutriates produced from a 24 h extraction cycle, and chemical analysis indicated low concentration of a complex mixture of hydrocarbon and metal contaminants. In contrast, the same elutriates impaired reproduction and growth after a 21-day chronic exposure. F1 neonates from the chronic tests were tested for sensitivity to the reference substance potassium dichromate, revealing a decrease in their sensitivity. Inter-generational effects were also observed, with a significant decrease in subsequent neonate production, when daphnids were moved to a clean medium. Supplemental acute toxicity assays using 48 and 72 h bitumen extraction cycles progressively increased daphnid mortality after a 48-h exposure to the respective elutriates. This indicates that bitumen-associated contaminants are being liberated after initial input and fluvial washing (24 h), highlighting the need for future work to assess toxicity responses and associated elutriate water chemistry of a longer fluvial exposure time-series. This work contributes to our understanding of the possible effects of natural bitumen exposure on riverine aquatic ecosystems, providing new information to inform the delineation of baseline conditions to assess environmental change and the design of future regional effects-based monitoring programs.

1 - Introduction

The Athabasca River and its associated major tributaries (the Clearwater, Ells, Steepbank, Muskeg, and Firebag Rivers) drain approximately 160,000 km^2 of north-eastern Alberta, Canada with a significant portion of the lower basin flowing through the Athabasca oil sands geological deposit. The Athabasca deposit comprises approximately 33% of the total oil sands formation that occur beneath 142,200 km^2 of land surface in the region, with approximately 4,800 km^2 being sufficiently shallow to make commercial-scale surface mining viable (Akre et al., 2004; Alberta, 2019; Conly et al., 2002; Quagraine et al., 2005). The main stem of the Athabasca River and the lower portions of contributing tributaries incise and flow through these shallow oil sands bitumen outcrops, creating a natural source of sediment-associated hydrocarbons and other chemical contaminants to the aquatic environment through fluvial erosional and deposition processes (Akre et al., 2004; Barton and Wallace, 1979; Conly et al., 2002; Droppo et al., 2019, 2018; Evans et al., 2019; Headley et al., 2001; Headley and McMartin, 2004). The combination of the surface and groundwater flows through the oil sands geological formation coupled with episodic high flow events that can slump, transport and deposit relatively large volumes of oil-sands sediments poses an exposure risk to the aquatic systems due to the potential high load of associated contaminants and related physical habitat disturbance (Conly et al., 2002; Droppo et al., 2018, 2019; Evans et al., 2019; Gibson et al., 2016) (Figures 1a,b). Given the complexity of the potential sources, magnitude and duration of oil sands sediment-associated contaminants entering the riverine ecosystems in the Athabasca region, there is a need for a more comprehensive understanding of the potential stressors, exposure pathways, and associated toxicological and ecological impacts and risks on aquatic organisms (Arciszewski et al., 2017; Droppo et al., 2018, 2019; Evans et al., 2019).

To date, only a limited number of studies have examined the ecotoxicological effects on aquatic organisms of direct or indirect exposure to natural oil sands sediments. Tetreault et al. (2003) reported changes in biochemical parameters in fish residing in the natural oil sands deposit when

compared to the same fish species residing in reference conditions outside of the deposit. Moreover, the adverse effects increased with the proximity to areas having active mining activities. In a laboratory exposure study, Colavecchia et al. (2004) reported significant hatching alterations in early life stages of the fathead minnow *Pimephales promelas*, with increased mortality, reduced body size, and larval deformities when exposed to either natural and anthropogenic-derived oil sands material. In a subsequent study, Colavecchia et al. (2006) found that exposure to riverine oil sands sediments impaired the eggs and larval development and survival of the white sucker *Catostomus commersoni*. Vignet et al. (2019) examining 21-d chronic exposure of fathead minnow eggs and larvae to Steepbank and Ells river bed sediments found significantly decreased survival, growth and tail length in the larvae. However, after a subsequent 5-month grow-out and recovery period when exposed to clean sediments, all initially observed growth impairment effects were reversed, except for exposure to the highest concentration of Steepbank river sediments.

Integrated studies by Droppo et al. (2018, 2019) and Evans et al. (2019) provide increased insights into the role of bitumen erosional outcrop processes and subsequent consequences on fluvial sediment and water column chemistry, bitumen associated contaminant (e.g., metals, Polycyclic Aromatic Compounds (PAC)) environmental exposure pathways, and related toxicological and ecological effects on aquatic biota. Geologically exposed bitumen beds containing high PAC concentrations are common to the majority of rivers and tributaries in the Athabasca oilsands region and related suspended fluvial sediments and associated contaminants can be transported long distances. Suspended sediment concentrations were found to increase downstream progressively with increasing discharge, and the associated PAC concentrations and chemical signatures were not homogenous across the geological formation with both petrogenic and pyrogenic (i.e., regional forest fire-related) characteristics evident (Droppo et al., 2018; Evans et al., 2019). Substantial PAC yield in the Ells River resulting from high total eroded sediment produced the highest toxicity from both parental and eroded sediment on fathead minnow larval development compared with

sediments from the Steepbank River (Droppo et al., 2019). Evans et al. (2019) found PAC concentrations in river sediments to vary three orders in magnitude from headwater regions to downstream areas and were highest at tributary mouths and along reaches of the Athabasca River having exposed bitumen formations. Water PAC concentrations were found to be less variable, but were also higher in river reaches near exposed bitumen beds. Low-molecular-weight PACs, particularly naphthalenes and fluorenes, generally dominated the classes of PACs found in tissues of forage fish in the region, while phenanthrenes were in higher concentrations in fish caught in areas with proximity to bitumen outcrops.

The present study further advances our understanding of aquatic organism exposure to bitumen bearing eroded river-bank soils by assessing the acute and chronic ecotoxicological effects using the *Daphnia magna* bioassay. This bioassay is an internationally recognized standard for assessing acute and chronic ecotoxicology effects of toxic substances in freshwater systems to inform regulatory decision-making (Biesinger et al. 2002; Environment Canada 1990, 1996; OECD 2004, 2012). We examine exposure to conditions that simulate early stages of erosional input of river-bank soils into the aquatic receiving environment on a range of *D. magna* life history endpoints, including survival, reproduction, F1 generation health status, and recovery from exposure. Results from this study will further advance the capability to refine the quantification of regional environmental baseline conditions and contributes to improving the discrimination of the environmental risks and effects of exposure to natural versus anthropogenic-derived oil sands contaminants in aquatic environments.

2 - Material and methods

2.1 – Bitumen Soil Source

Three replicate samples (STB-CF1, STB-CF2, STB-CF3) of parental bitumen-bearing soils were taken approximately 5 meters apart from a slumping region of the bank of the lower reach of the

Steepbank River (STB), Alberta, Canada (56° 58.754' N 111° 17.902' W). The site is located at the interface between the Clearwater and McMurray oil sands geological formations and outside the zone of the direct influence of the oil sands open pit mining development (Tetreault et al. 2003, Droppo et al. 2018, 2019). The samples were packed in separate food-grade plastic bags under refrigerated conditions and stored at 4° C until shipped to the Department of Biology, University of Aveiro, Portugal. The samples were taken from the same field location used by Droppo et al. (2019) (Steepbank-Clearwater formation site; STB-CF), thereby allowing for the comparability of results.

To produce the experimental elutriate exposure series, the three individual field samples were treated as independent treatments and not used to create a composite, homogeneous sample. This was due to apparent observed differences in the physical properties in each source sample (i.e., differing proportional aggregates of sand, clays, and bitumen) and challenges associated with using mechanical mixing methods.

2.2 – Elutriate Extraction

Upon arrival, the river bank bitumen soil samples continued to be stored at 4° C at the University of Aveiro until use for elutriate extraction. Standardized 24 h elutriate extractions were produced for each field sample of bitumen-bearing riverbank soils (STB-CF1, STB-CF2, STB-CF3), following van Gestel et al. (2001), Baun et al. (2002), Loureiro et al. (2005), and Sforzini et al. (2016) who developed protocols to ensure reliable, reproducible and representative extraction methods for use in acute and sublethal bioassays of contaminated soils. Elutriate extraction was performed using a 1:2 ratio (solid: liquid / 400g of bitumen bearing soil: 800 ml of ASTM per extraction process), mixing bitumen with artificial moderate hard water (ASTM, 1980). The mixture was shaken in the dark for 24 h on a benchtop orbital shaker and afterward placed in 50 mL *Falcon* tubes for centrifugation (45 min, at 3220g).

Supplementary elutriates (48 and 72 h) were performed to assess whether longer extraction times simulating extended fluvial washing processes produce different toxicity results from those attained using the standard 24 h extraction protocol outlined above. After the collection of the supernatant from a 1st extraction cycle (Elutriate 1; 24 h wash), the remaining sediment pellet was used for a new cycle of extraction (2nd cycle – Elutriate 2; 48 h wash), using the same methodology as previously described for the 1st cycle. This procedure was repeated to obtain the 3rd cycle (Elutriate 3), resulting in an extraction series spanning 72 hours.

All elutriates were stored at 4° C until testing, never exceeding a one-week holding time. Also, since the bitumen elutriate samples were not clear optically, possible turbidity effects associated with the bitumen elutriate treatments were assessed against a control treatment using the natural standard sandy-loam soil LUFA 2.2 (Speyer, Germany), where a non-contaminated turbid sample was tested, according to Loureiro et al. (2015).

2.3 – Experimental Design and *Daphnia magna* bioassays

2.3.1 – Experimental Design

Figure 2 outlines the overall experimental design of the elutriate exposure studies. Acute and chronic ecotoxicological tests using the K6 clone of *Daphnia magna* Straus (originally from Antwerp, Belgium) were performed for each elutriate combination, as described below. The K6 clone has been kept in laboratory culture for more than ten years at the Department of Biology, University of Aveiro, Portugal. Cultures were maintained in ASTM moderated-hard-water medium on a controlled temperature regime (19 - 21 °C) at a 16 h light–8 h dark photoperiod. Daphnids in cultures were fed every two days with *Raphidocelis subcapitata* (formerly known as *Pseudokirchneriella subcapitata*) at a concentration of $3x10^5$ cells/ml and supplemented with an organic extract (Marinure seaweed

extract, supplied by Glenside Organics Ltd.) (Baird et al., 1989). For testing, neonates used were selected from the 3rd to 5th broods.

2.3.2 – Acute tests – *Daphnia magna* Immobilisation test

Non-diluted elutriates (100% of elutriate) were used in a ratio of 5 animals per replicate, in a set of 5 replicates per treatment, at 20±1 °C with a 16:8h light: dark photoperiod, following the immobilization test OECD 202 guideline (OECD, 2004) (Figure 2). Negative control of ASTM and an elutriate control collected from natural LUFA 2.2 soil (following the same procedure for elutriate extraction described above, using LUFA 2.2 instead of bitumen samples mixed with ASTM) were used in the acute toxicity tests. To check the immobilization of daphnids when in the presence of oil sands elutriates, each replicate had 50 mL of the respective media (controls and pure elutriates), and daphnids were observed after 24h and 48h of exposure, where the number of immobilized organisms was recorded. No food was provided during the test.

2.3.3 – Chronic tests – Reproduction of *Daphnia magna*

The reproductive capacity of *D. magna* was evaluated during the exposure to the 24h extraction elutriate for all three bitumen samples (STB-CF1, STB-CF2, STB-CF3). Each elutriate treatment had ten replicates, with one neonate each (<24h old; 3rd to 5th brood), negative control of ASTM and an elutriate control of LUFA 2.2 soil complemented the control treatments. Organisms were fed daily with *R. subcapitata* ($3x10^5$ cells/ml, plus organic extract) under a 16:8 h light: dark photoperiod regime at 20±1 °C and were exposed in a final volume of 50 ml. The test ran for 21 days and the treatment elutriates were replenished every two days along with the controls, according to the OECD 211 guideline (OECD, 2012). During the test, survival and the total number of neonates were

recorded, along with parental body length (mm), measured from the head to the insertion of the anal spine under a stereomicroscope, after the 21 days of exposure.

2.3.4 – Sensitivity test and recovery of F1 generation daphnids

To assess the health status of the produced neonates (generation F1), organisms from the 5th brood (< 24h old) were collected from the chronic test (section 2.3.3) and were exposed to a range of $K_2Cr_2O_7$ concentrations (0.3 – 3.0 mg/kg) for 24h, using similar methodologies as described above (2.3.2), with immobilized organisms being recorded (Figure 2). Neonates from the 5th brood were selected as this generation of neonates coincided with the reproductive cohort arising after 21-day chronic exposure. $K_2Cr_2O_7$ is used as a reference substance following the OECD 202 guideline (OECD, 2004) as an international ring-test used to assess fitness in daphnids. F1 neonates from all exposures (from both negative and positive controls, plus elutriates from STB-CF1, STB-CF2, STB-CF3) were also used in a reproduction test, exposed to ASTM media to assess the recovery of neonates in clean medium (Figure 2). The test procedures were similar to those described above for the reproduction test. The number of neonates produced was recorded.

2.3.5 Supplementary Elutriate Exposure

Supplementary elutriate extractions (48 and 72 h) and associated 48 h exposure acute toxicity bioassays were performed using *D. magna* to assess whether longer extraction times simulating extended fluvial washing processes produce different results from those attained using the standard 24 h contaminated soil extraction protocol (see section 2.2 and Figure 2).

2.4 – Elutriate Chemical Analysis

Chemical characterization of metals, parent and alkylated PACs and Naphthenic Acids (NA) was conducted only on the solid phase of the STB-CF3 bitumen soil sample and the corresponding 24 h extraction elutriate. The rationale for this was based on the result that the STB-CF3 sample induced the most substantial chronic effects on daphnids, with a larger reduction in the total number of neonates produced and corresponding decreased daphnid growth. The bitumen sediment sample and elutriate were analyzed for metals and Naphthenic Acids (NAs) by InnoTech Alberta, Canada and the parent and alkylated PACs by SGS AXYS Analytical Services Ltd., Sidney, BC, Canada.

NAs aqueous solutions were analyzed using HPLC-Orbitrap-MS in water samples, previously adjusted to pH≈2, spiked with international (Dodecanoic acid-d23) and extracted by automated solid-phase extraction. Compound characterization and quantification were performed using liquid chromatography coupled to Orbitrap mass spectrometer. NAs were analyzed in solid bitumen samples with a previous liquid/liquid extraction with an alkaline solution before analysis using HPLC-Orbitrap-MS. Metals were analyzed by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) (Elan DRC-II with ESI SC-8XC high throughput FAST autosampler). Before analysis, the digestion of samples was performed using an Ethos UP with the Maxi44 rotor (Milestone Inc).

The AXYS Analytical Method MLA-021 (Rev 10) was used for the determination of concentrations of PACs (both alkylated and parent) in the elutriate and bitumen sediment samples. This method uses a high-resolution gas chromatography/low-resolution mass spectrometry (HRGC/LRMS) performed on an Agilent 6890N GC/5973 MS/7683 Autosampler (SGS AXYS Analytical Ltd, Sidney, BC, Canada). SGS AXYS Ltd. is accredited by the Canadian Association for Laboratory Accreditation Inc. (CALA) (Lab ID A2637) in Canada for the analysis of PAHs in aqueous media and solids.

2.5 – Statistical analysis

Data normality and equality of variances were tested by the Shapiro-Wilk and Levene´s tests, respectively. Student t-tests were performed to assess statistical differences between the ASTM control and the elutriate control LUFA 2.2 treatments. One-way ANOVA followed by a multiple comparison procedure (post hoc Tukey test) were used to test for significant differences ($p<0.05$) in immobilization, the total number of neonates per female, and length of daphnids among the three (STB-CF1, STB-CF2, STB-CF3) treatment elutriate groups, controls, and recovery tests. The sensitivity of daphnid exposure was assessed by calculating the concentration that caused 50% of survival on the daphnid population (LC_{50}) using probit analysis (MINITAB 14).

3 - Results and discussion

3.1 – Ecotoxicology of elutriates

The turbidity visible to the unaided eye of LUFA 2.2 elutriates did not affect the performance of *Daphnia magna* (t-test; $p>0.05$), achieving one of the validation criteria in elutriates extraction.

No significant acute lethality (mean % survival) was observed in F0 daphnids exposed for 48h to the 24 h elutriate extraction of STB-CF1, STB-CF2 and STB-CF3 bitumen samples (One-way ANOVA; $p>0.05$) (Figure 3).

Chronic toxicity tests performed using the 24 h elutriates from the three sample sites revealed significant impairment in daphnids reproduction during a 21d exposure and reduction in the total neonates produced per female (F1 neonates) (Tukey test; $p<0.05$) (Figure 4). The ANOVA results also revealed statistical differences in all three field samples compared with both the ASTM and LUFA 2.2 exposure controls ($p<0.05$). Both STB-CF1 and STB-CF2 samples differed significantly from STB-CF3, which had a significantly lower total number of neonates per female (Figure 4; Tukey test, $p<0.05$). Daphnid growth (measured as body length) was found to differ from the Lufa 2.2 elutriate control

significantly for all three sites (STB-CF1, STB-CF2, STB-CF3), while only STB-CF3 differed from all other samples and controls (Figure 5; Tukey test, $p<0.05$).

In the sensitivity test with the reference substance $K_2Cr_2O_7$, F1 neonates collected from parental daphnids exposed to natural bitumen elutriates were more sensitive than those from the two controls (ASTM and LUFA 2.2 soil elutriates) Comparing the LC_{50}s, all the observed values were in the recommended range provided by the OECD (2004) guideline (0.6 mg/L to 2.1 mg/L). Nevertheless, the observed LC_{50}s of daphnids from both controls (1.59 and 1.84 mg/L) were more than twice the LC_{50}s of the daphnids pre-exposed to oil sands elutriates (0.63 – 0.65 mg/L). This suggests that after exposure to bitumen associated contaminants, daphnids were more sensitive than the ones not pre-exposed to the treatment elutriates.

After the 21 days of exposure, daphnids from the 5th brood in the reproduction assay were subsequently used in the reproduction recovery tests (using ASTM media only). While a slight recovery was observed regarding the total neonates produced per female (Figure 6), statistical differences were still found between pre-exposed organisms and controls ($p<0.05$).

The acute bioassays performed using the supplementary 48 and 72 h elutriate extractions revealed stronger toxicological effects with longer bitumen washing/extraction cycles (Figure 7). While the 48 h extraction produced similar levels of toxicity (mean survival %) to F0 individuals exposed to the 24 h elutriate (Figure 7A), a significant decrease was observed in daphnid survival in all three bitumen source samples when exposed to the 72 h elutriate (Tukey test, $p<0.05$; Figure 7B). This indicates that prolonged watershed-related fluvial/erosional processes could be responsible for the additional liberation of bitumen-bound contaminants at levels toxic to aquatic organisms.

3.2 – Chemical Analyses

Table 1 summarizes the metal concentrations of the riverbank soil sample and associated 24 h elutriate extraction from the STB-CF3 replicate. The primary metals associated with the sample were aluminium (46%), iron (41%) and calcium (10%). The primary metal constituents in the corresponding elutriate were calcium (89%) and chloride (7%) in both the total and dissolved forms. All concentrations of metals in the elutriates, both total and dissolved, are below the reported US EPA LC_{50} values based on *D. magna* 48 h acute toxicity tests and are below or within the Canadian Council of Ministers of the Environment (CCME) long-term water quality guidelines for the protection of aquatic life. Parental soil material and the elutriate had total metal concentrations of ∑ metal 286367 µg/g and 283712 µg/L, respectively.

Table 2 summarizes the concentrations of parental non-alkylated and alkylated PACs found in the STB-CF3 bitumen sample and the associated 24 h elutriate extraction. Although in this study the PAC analysis was performed only on the STB-CF3 treatment sample, the results are consistent with other analyses reported on related sediment and water samples from the region (Conly et al., 2002; Droppo et al. 2018, 2019; Evans et al., 2019). The composition of the PACs in the STB-CF3 24 h elutriate was dominated (~ 80% relative proportion) by low molecular weight (three or fewer carbon rings) classes (naphthalenes, biphenyls, phenanthrenes), followed by medium- to high- molecular weight PACs (> four carbon rings: fluorenes (7%), fluoranthenes (6%) and dibenzothiophenes (5%)). None of the concentrations exceeded the US EPA LC_{50} values for *D. magna* 48 h acute toxicity or the CCME PAH long-term water quality guidelines for the protection of aquatic life where available. By comparison, the dominant ΣPAC composition of the solid parental bitumen sample consisted of dibenzothiophenes (26%), naphthalenes (24%), fluoranthenes (17%), phenanthrenes (10%), biphenyls (9%) and anthracenes (6%), which is characteristic of compounds associated with bitumen hydrocarbon sources (Conly et al., 2002; Droppo et al., 2018, 2019; Evans et al., 2019). The total ΣPAC concentration in the 24 h elutriate was 207 ng/L, which is consistent with the higher end of the range of field values reported by Evans et al. (2019) for water quality samples from the mainstem of

the Athabasca River (ranging from a low of 87 ng/L to a high of 217 ng/L) and is similar to the lower range of ΣPAC concentrations reported for the Steepbank River. Water ΣPAC values from the Steepbank river ranged from a high of 4667 ng/L to a low of 110 ng/L between two sampling years (2012-2013), highlighting a high level of inter-annual variability that was likely associated with greater inputs of PACs eroded from exposed bitumen outcrops/soils in 2012, but also reflective of among-site gradient differences within the watershed related to the proximity and exposure to bitumen outcrops (Evans et al., 2019). Total naphthenic acid concentrations were 33.4 µg/g and 28.6 µg/L in the solid and elutriate samples, respectively.

3.3 – Summary

As highlighted by Conly et al. (2002), Headley et al. (2001), Headley and McMartin (2004), and more recently by Frank et al. (2014), Gerner et al. (2017), Droppo et al. (2018, 2019), Evans et al. (2019) and Vignet et al. (2019), the discrimination between natural and anthropogenic sources of contamination in the oil sands area is a challenging but crucial to objectively defining baseline ecological conditions against which environmental change can be assessed. Fluvial erosion and deposition of naturally occurring bitumen deposits into riverine surface waters along with other oil-sands related contaminant input sources (e.g., atmospheric deposition of oil sands emissions, regional surface water - groundwater interactions) cumulatively pose potential risks to the health of aquatic organisms, with cascading implications to riverine aquatic ecosystem structure and function. Regional monitoring and associated research efforts need to continue to focus on quantifying the relative contributions of the various oil sands related contaminant sources, associated exposure pathways and resulting toxicological and ecological effects at appropriate spatial and temporal scales (Arciszewski et al., 2017).

This study contributes new information on the potential ecotoxicological effects of natural fluvial inputs of river-bank eroded bitumen soils on aquatic biota using *Daphnia magna* when exposed to elutriates derived from simulated fluvial processing/washing of bituminous parental material. While no significant acute lethality was observed in *D. magna* adults when exposed for 48h to a 24 h extraction elutriate from the bitumen samples (Figure 3), in contrast, the 21-day chronic toxicity bioassays revealed significant impairment in daphnid reproduction and a reduction in the total neonates produced per female (F1 neonates) (Figures 4, 5). In addition, statistical differences were observed among all three field samples compared with both the ASTM and LUFA 2.2 exposure controls (Figures 4, 5). Surprisingly, heterogeneity of chronic toxicity responses was also observed among the three source bitumen soil samples, even though the replicates were taken only 5 meters apart at the river-bank field site. This further reinforces the results of Droppo et al. (2018, 2019) and Evans et al. (2019) and highlights the hydrological, geological and fluvial geomorphological complexity that needs to be considered in future monitoring and ecotoxicological assessment designs when evaluating the effects of naturally occurring bitumen associated soils entering the watershed and subsequent fluvial sediment exposure on aquatic species.

Although the presence of generally low concentrations of aqueous PACs and metal contaminants in the representative STB-CF3 24 h elutriate and none were found to exceed environmental guidelines (Tables 2, 3), chronic toxicological effects were observed (Figures 4-6). Moreover, chronic, long-term exposure to the complex mixture of aqueous PACs and metals in the elutriates revealed potential population-level effects, as highlighted by the low recovery by the F1 individuals and lower growth rates of *Daphnia* adults (Figures 5, 6).

The supplementary elutriate extraction series and associated acute toxicity tests further revealed that associated bitumen contaminants are released with time, potentially increasing exposure risks to aquatic biota. Elutriates produced after 72h of bitumen soil washing from all three sampling locations (STB-CF1, STB-CF2, STB-CF3) had significantly higher acute toxicity (observed decrease in

mean survival %) in *D. magna* adults than when exposed to elutriates generated from the 24 and 48h extractions (Figure 7). This highlights that the standard 24 h extraction protocol as recommended by toxicity studies where elutriates are produced from soil/solid samples (e.g., van Gestel et al., 2001; A. Baun et al., 2002; Loureiro et al., 2005; Sforzini et al., 2016) may not properly represent the time-series of contaminant release and related potential acute and chronic exposure toxicological effects. The lack of toxicity response observed by Droppo et al. (2019) who used a 2 h bitumen wash cycle to produce their exposure elutriate may be reflective of insufficient extraction time and related bitumen contaminant concentrations to produce any effects the hatching success of fathead minnow embryos.

Collectively these studies highlight the importance of conducting further research into the refinement of the time-dependent release of contaminants associated with the input and subsequent hydrological processing (scour and washing) of natural bitumen containing soils and sediments. The increase in acute toxicity observed in this study with the longer bitumen wash cycle indicates the need for a longer time series simulation of the fluvial weathering processes of the bitumen source material, complemented with associated chemical profiling of the elutriates along with both acute and chronic toxicity tests. This information is fundamental to informing a risk-based approach to addressing the ecotoxicological impacts of sediment-associated exposure.

This study also reinforces the need for longer-term multi-generational chronic ecotoxicological tests to provide more realistic and conclusive information regarding population-level responses and resulting cascading ecological effects arising from exposure to naturally occurring bitumen sources in the Alberta oil sands region. Defining ecological baseline conditions in the oil sands region must be informed on having a more detailed understanding of the geographic extent and magnitude of effects that exposure to natural oil sands deposits and related fluvial sediments have on aquatic biota.

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Assessing the acute and chronic toxicity of exposure to naturally occurring oil sands deposits to aquatic organisms using *Daphnia magna*.

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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Figure 1a – River bank slumping of natural bitumen outcrops in the Steepbank River, Alberta, Canada.

Figure 1b – Natural bitumen sample collected in June 2014 at the Athabasca Basin (Coordinates: 56° 58.754' N 111° 17.902' W), on the banks of the Steepbank River, Alberta, Canada, approximately at the interface between the Clearwater and McMurray geological formations.

Figure 2 – Experimental design for the ecotoxicological evaluations for the three elutriate extraction cycles derived from naturally occurring oil sands bitumen samples from the Steepbank River, Alberta (STB-CF1, STB-CF2, STB-CF3). A recovery/sensitivity bioassay was also conducted using only the first extraction cycle (Elutriate 1) for all three sampling sites.

Figure 3 – Mean survival (%) and associated standard error of *Daphnia magna* exposed for 48h to elutriates produced from naturally occurring oil sands bitumen samples from the Steepbank River, Alberta (STB-CF1, STB-CF2, STB-CF3). Elutriates were produced after 24h of extraction. Analysis of variance showed no significant differences among treatments ($p<0.05$).

Figure 4 –Reproduction output (mean total number of neonates per female with standard error) of *Daphnia magna* exposed for 21 days to elutriates produced from 24 h of extraction of naturally occurring oil sands bitumen samples from the Steepbank River, Alberta (STB-CF1, STB-CF2, STB-CF3). Ct represents the ASTM and Lufa 2.2 the control elutriates. Analysis of variance showed significant differences among control and bitumen elutriate samples ($p<0.05$) treatments. A post-hoc Tukey test showed the controls (a) differed significantly from the bitumen elutriates (b,c), and STB-CF3 showed significantly lower neonate production (c) than the other two bitumen samples (b).

Figure 5 – Length of parental *Daphnia magna* (mm) after a 21-day exposure to elutriates produced from 24 h of extraction of naturally occurring bitumen bearing soil samples from the Steepbank River, Alberta (STB-CF1, STB-CF2, STB-CF3). Ct represents the ASTM and Lufa 2.2 the control elutriates. Analysis of variance showed significant differences among control and bitumen elutriate samples ($p<0.05$). A post-hoc Tukey test showed the controls (a) differed significantly from the bitumen elutriates (b,c), although the Ct control was not significantly different from STB-CF1 and STB-CF2. STB-CF3 showed significantly lower parental lengths (c) than the other two bitumen samples (b).

Figure 6 – Reproductive output (mean total number of neonates per female and standard error) of F1 *Daphnia magna* during the recovery test for 21 days in ASTM media. Organisms were pre-exposed to Ct, Lufa 2.2, STB-CF1, STB-CF2 and STB-CF3 elutriates. Analysis of variance showed significant differences among control and bitumen elutriate samples ($p<0.05$). A post-hoc Tukey test showed the controls (a) differed significantly from the bitumen elutriates (b,c), although the Lufa 2.2 control was not significantly different from STB-CF3. The STB-CF1 and STB-CF2 treatments showed significantly lower reproductive output (c) than STB-CF3 (b).

Figure 7 – Mean survival (%) and associated standard error of *Daphnia magna* exposed for 48h to elutriates produced from naturally occurring oil sands bitumen samples from the Steepbank River, Alberta (STB-CF1, STB-CF2, STB-CF3). Elutriates were produced after (A) 48h and (B) 72h of extraction. Ct represents the ASTM and Lufa 2.2 the control elutriates. Analysis of variance showed no significant differences among treatments for 48h elutriates (A). Significant differences among treatments and controls were observed for 72h elutriates (B); a post-hoc Tukey test showed the controls (a) differed significantly from the bitumen elutriates (b) ($p<0.05$), although no differences were observed among the sample sites.

Table 1 – Total and dissolved metal concentration levels from the STB-CF3 24 h elutriate and associated source riverbank bitumen sample from the Steepbank River, Alberta, Canada. LC_{50} values are the lowest 48 h *Daphnia magna* acute toxicity value reported from the U.S. Environmental Protection Agency ECOTOXicology Knowledgebase System (https://cfpub.epa.gov/ecotox/), when available. All measured elutriate metal concentrations (Total and Dissolved) are below US EPA LC_{50} values and are below or within the Canadian Council of Ministers of the Environment (CCME: http://st-ts.ccme.ca/en/index.html) water quality guidelines for the protection of aquatic life (* calculated from equations based on pH (7.5-8.2) / $CaCO_3$ (130-160) mg/L Hardness ranges for ASTM exposure medium).

	STB-CF3; 24 h elutriate (µg/L)		**STB-CF3; Solid parental sample (µg/g)**	***D. magna* 48 h LC_{50} (µg/L)**	**CCME Water Quality Guideline (µg/L)**
	Total	Dissolved	Total		
Aluminium	7.8	0.75	133000	38000	**-**
Antimony	0.577	0.569	1.05	18000	**-**
Arsenic	0.386	0.381	21.7	3800	**5**
Barium	27.3	26.8	552	410000	**-**
Beryllium	0.030	0.030	3.62	1000	**-**
Bismuth	0.023	0.023	0.485	-	**-**
Boron	1740	1730	250	-	**1500**
Cadmium	0.082	0.081	0.247	24	**0.37**
Calcium	256000	255000	30900	-	**-**
Chloride	22000	21600	415	-	**-**
Chromium	0.04	< 0.1	85.5	22	**-**
Cobalt	0.272	0.067	34.1	400	**-**
Copper	4.35	3.77	70.3	24	**2.96-4 ***
Iron	15.4	1.4	116000	76000	**300**
Lead	0.080	0.054	29.4	400	**4.44-5.79 ***
Lithium	261	261	125	-	
Manganese	2.95	0.58	723	29000	**260-570 ***
Molybdenum	1.90	1.86	2.12	-	**73**
Nickel	12.4	11.7	83.1	740	**117-137 ***
Selenium	3.73	3.68	2.07	430	**1**
Silver	0.022	0.021	0.743	1.5	**0.25**
Strontium	5150	5060	376	-	**-**
Thallium	0.0642	0.0634	0.810	-	**0.8**
Thorium	0.0493	0.0487	15.7	-	**-**
Tin	0.032	0.031	2.00	-	**-**
Titanium	1.23	0.80	3340	-	**-**
Uranium	1.16	1.14	3.54	-	**15**
Vanadium	0.31	0.28	168	1550	**-**
Zinc	8.3	7.65	162	68	**8.9-17**
∑ Metals	**285239**	**283712**	**286367**		

Table 2 - Polycyclic Aromatic Compounds (PACs) concentrations in the STB-CF3 24 h elutriate and source riverbank bitumen soil sample analyzed using gas chromatography-mass spectrometry (GC-MS), with associated relative percentage composition by each PAC class and by total PACs. LC_{50} values are the lowest 48 h *Daphnia magna* acute toxicity values reported in the U.S. Environmental Protection Agency ECOTOXicology Knowledgebase System (https://cfpub.epa.gov/ecotox/), when available; <D.L. – below the detection limited. All elutriate PAC concentrations are below the U.S. EPA LC_{50} toxicity values and where available, the Canadian Council of Ministers of the Environment (CCME: http://st-ts.ccme.ca/en/index.html) water quality guidelines for the protection of aquatic life. $^{\tau}$ US-EPA High Priority Pollutants (**in bold**) - these 16 PAHs are of environmental concern because of their potential toxicity in humans and other organisms and their prevalence and persistence in the environment.

PAC class	STB-CF3 - elutriate		STB-CF3 – Solid parental sample		*D. magna* 48 h LC_{50} (µg/L – EPA)	CCME Guideline (ng/L)
	Concentration (ng/L)	% contribution	Concentration (ng/g)	% contribution		
Naphthalene (NAP)$^{\tau}$	22.7	10.97	102	5.02	3400	1100
C_1-Naphthalenes	15.7	7.59	146	7.18	-	-
C_2-Naphthalenes	23.6	11.40	<D.L.	0.00	-	-
C_3-Naphthalenes	9	4.35	34.1	1.68	-	-
C_4-Naphthalenes	1.67	0.81	50.3	2.48	-	-
1-Methylnaphthalene	5.37	2.59	55.7	2.74	-	-
2-Methylnaphthalene	10.3	4.98	90.8	4.47	-	-
1,2-Dimethylnaphthalene	<D.L.	0.00	<D.L.	0.00	-	-
2,6-Dimethylnaphthalene	1.87	0.90	<D.L	0.00	-	-
2,3,5-Trimethylnaphthalene	<D.L.	0.00	<D.L.	0.00	-	-
2,3,6-Trimethylnaphthalene	0.972	0.47	<D.L.	0.00	-	-
1,4,6,7-Tetramethylnaphthalene	<D.L.	0.00	<D.L.	0.00	-	-
Total concentration of PACs within class and % sample class contribution relative to ∑ all PACs	**91.182**	**44.06 %**	**478.9**	**23.56 %**		
Fluorene (FLU)$^{\tau}$	1.92	0.93	<D.L.	0.00	-	3000
C_1-Fluorenes	2.84	1.37	<D.L.	0.00	-	-
C_2-Fluorenes	2.66	1.29	<D.L.	0.00	-	-
C_3-Fluorenes	6.47	3.13	<D.L.	0.00	-	-
2-Methylfluorene	<D.L.	0.00	<D.L.	0.00	-	-

1,7-Dimethylfluorene	<D.L.	0.00	<D.L.	0.00	-	-
Total concentration of PACs within class and % sample class contribution relative to ∑ all PACs	**13.89**	**6.71 %**	**0**	**0.00 %**		
Acenaphthene (ACE)$^{\tau}$	<D.L.	0.00	<D.L.	0.00	120	5800
C_1-Acenaphthenes	<D.L.	0.00	<D.L.	0.00	-	-
Total concentration of PACs within class and % sample class contribution relative to ∑ all PACs	**0**	**0%**	**0**	**0.00 %**		
Phenanthrene (PHEN)$^{\tau}$	7.49	3.62	40	1.97	700	400
C_1 Phenanthrenes/Anthracenes	2	0.97	<D.L	0.00	-	-
C_2 Phenanthrenes/Anthracenes	2.15	1.04	40.3	1.98	-	-
C_3-Phenanthrenes/Anthracenes	1.13	0.55	24.7	1.22	-	-
C_4-Phenanthrenes/Anthracenes	3.57	1.72	91.7	4.51	-	-
3-Methylphenanthrene	1.17	0.57	<D.L.	0.00	-	-
2-Methylphenanthrene	0.828	0.40	<D.L.	0.00	-	-
9/4-Methylphenanthrene	<D.L.	0.00	<D.L.	0.00	-	-
1-Methylphenanthrene	<D.L.	0.00	<D.L.	0.00	-	-
3,6-Dimethylphenanthrene	0.255	0.12	<D.L.	0.00	-	-
2,6-Dimethylphenanthrene	<D.L.	0.00	<D.L.	0.00	-	-
1,7-Dimethylphenanthrene	0.356	0.17	8.54	0.42	-	-
1,8-Dimethylphenanthrene	<D.L.	0.00	<D.L.	0.00	-	-
1,2,6-Trimethylphenanthrene	<D.L.	0.00	<D.L.	0.00	-	-
Total concentration of PACs within class and % sample class contribution relative to ∑ all PACs	**18.949**	**9.16 %**	**205.24**	**10.10 %**		
Anthracene (ANTH)$^{\tau}$	<D.L.	0.00	<D.L.	0.00	-	12
Benz[a]anthracene (B[a]A)$^{\tau}$	0.412	0.20	<D.L.	0.00	97.5	18
Dibenz[a,h]anthracene (D[ah]A)$^{\tau}$	<D.L.	0.00	<D.L.	0.00	-	-
2-Methylanthracene	<D.L.	0.00	<D.L.	0.00	-	-
C_1-Benzo[a]anthracenes/Chrysenes	0.81	0.39	35.3	1.74	-	-
C_2-Benzo[a]anthracenes/Chrysenes	0.742	0.36	68.6	3.38	-	-
C_3-Benzo[a]anthracenes/Chrysenes	<D.L.	0.00	13.5	0.66	-	-
C_4-Benzo[a]anthracenes/Chrysenes	<D.L.	0.00	<D.L.	0.00	-	-
Total concentration of PACs within class and %	**1.964**	**0.95 %**	**117.4**	**5.78 %**		

sample class contribution relative to ∑ all PACs						
Chrysene (CHRY)$^{\tau}$	1	0.48	17.8	0.88	-	-
5/6-Methylchrysene	<D.L.	0.00	<D.L.	0.00	-	-
1-Methylchrysene	<D.L.	0.00	9.42	0.46	-	-
5,9-Dimethylchrysene	<D.L.	0.00	<D.L.	0.00	-	-
Total concentration of PACs within class and % sample class contribution relative to ∑ all PACs	**1**	**0.48 %**	**27.22**	**1.34 %**		
Fluoranthene (FLTH)$^{\tau}$	2.34	1.13	13.7	0.67	78	40
Benzo[b]fluoranthene (B[b]F)$^{\tau}$	<D.L.	0.00	<D.L.	0.00	-	-
Benzo[j,k]fluoranthenes (B[j,k]F)$^{\tau}$	<D.L.	0.00	<D.L.	0.00	-	-
C_1-Fluoranthenes/Pyrenes	3.51	1.70	52.8	2.60	-	-
C_2-Fluoranthenes/Pyrenes	4.15	2.01	139	6.84	-	-
C_3-Fluoranthenes/Pyrenes	<D.L.	0.00	48.9	2.41	-	-
C_4-Fluoranthenes/Pyrenes	<D.L.	0.00	49.4	2.43	-	-
C_1-Benzofluoranthenes/Benzopyrenes	<D.L.	0.00	<D.L.	0.00	-	-
3-Methylfluoranthene/Benzo[a]fluorene	1.63	0.79	<D.L.	0.00	-	-
C_2-Benzofluoranthenes/Benzopyrenes	<D.L.	0.00	36	1.77	-	-
Total concentration of PACs within class and % sample class contribution relative to ∑ all PACs	**11.63**	**5.62 %**	**339.8**	**16.72 %**		
Pyrene (PYR)$^{\tau}$	1.39	0.67	**21**	**1.03**	130	25
Benzo[e]pyrene	<D.L.	0.00	<D.L.	0.00	-	-
Benzo[a]pyrene (B[a]P)$^{\tau}$	<D.L.	0.00	<D.L.	0.00	250	15
Indeno[1,2,3-c,d]pyrene (IND)$^{\tau}$	<D.L.	0.00	18.1	0.89	-	-
7-Methylbenzo[a]pyrene	<D.L.	0.00	<D.L.	0.00	-	-
Total concentration of PACs within class and % sample class contribution relative to ∑ all PACs	**1.39**	**0.67 %**	**39.1**	**1.92 %**		
Perylene	0.778	0.38	71.3	3.51	-	-
Benzo[g,h,i]perylene (B[ghi]P)$^{\tau}$	<D.L.	0.00	14	0.69	-	-
Total concentration of PACs within class and % sample class contribution relative to ∑ all PACs	**0.778**	**0.38 %**	**85.3**	**4.20 %**		
Biphenyl	11	5.31	67.6	3.33	2014	-
C_1-Biphenyls	9.59	4.63	86.1	4.24	-	-

C_2-Biphenyls	34.5	16.67	33.8	1.66	-	-
Total concentration of PACs within class and % sample class contribution relative to ∑ all PACs	**55.09**	**26.62 %**	**187.5**	**9.23 %**		
Dibenzothiophene	0.737	0.36	<D.L.	0.00	-	-
C_1-Dibenzothiophenes	<D.L.	0.00	<D.L.	0.00	-	-
2/3-Methyldibenzothiophenes	<D.L.	0.00	<D.L.	0.00	-	-
C_2-Dibenzothiophenes	1.5	0.72	52	2.56	-	-
2,4-Dimethyldibenzothiophene	<D.L.	0.00	<D.L.	0.00	-	-
C_3-Dibenzothiophenes	3.96	1.91	118	5.81	-	-
C_4-Dibenzothiophenes	4.9	2.37	360	17.71	-	-
Total concentration of PACs within class and % sample class contribution relative to ∑ all PACs	**11.10**	**5.36 %**	**530**	**26.08 %**		
Acenaphthylene (ACY)[τ]	<D.L.	0.00	<D.L.	0.00	-	-
Retene	<D.L.	0.00	21.8	1.07	-	-
∑ all PACs	**206.97**		**2032.26**			

Highlights

- Natural background evaluation in Oil sands is crucial for accuracy in risk assessment
- Bitumen elutriates induced a decrease in the fitness of *Daphnia magna*
- Heterogeneity of toxicity among the bitumen source samples
- Bitumen weathering increased the toxicity of elutriates

a

b

Figure 1

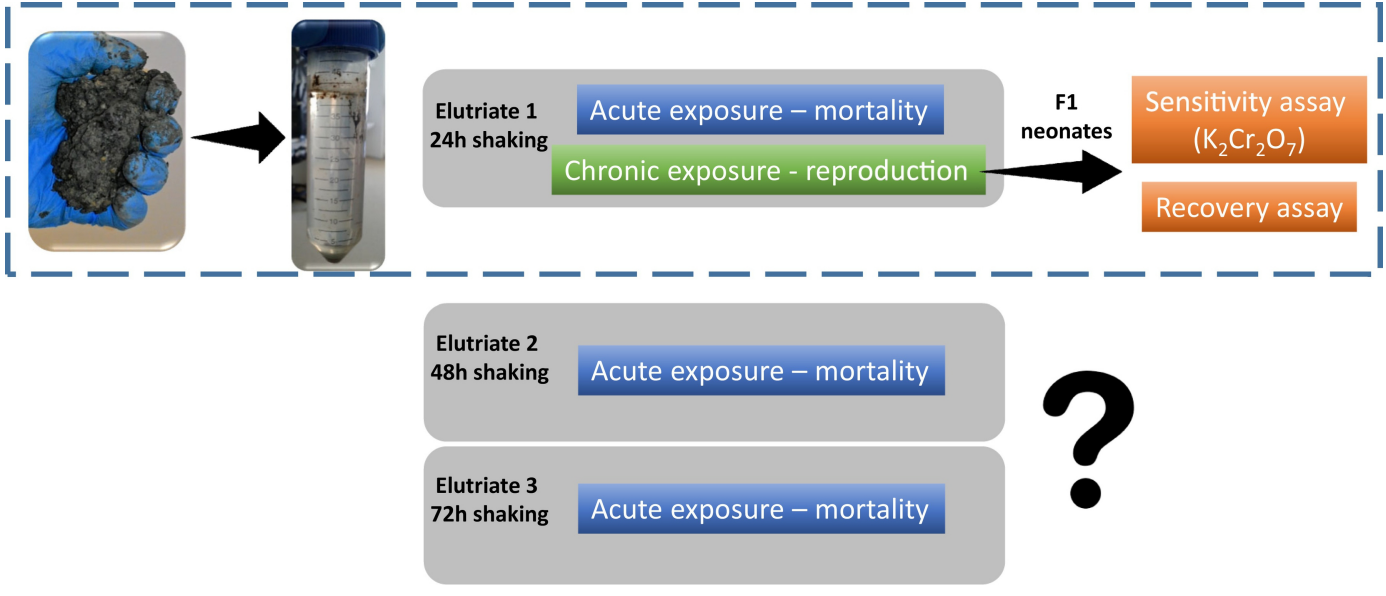
Elutriate 1
24h shaking
Acute exposure – mortality
Chronic exposure - reproduction
F1
neonates
Sensitivity assay
($K_2Cr_2O_7$)
Recovery assay
Elutriate 2
48h shaking
Acute exposure – mortality
Elutriate 3
72h shaking
Acute exposure – mortality
?

Figure 2

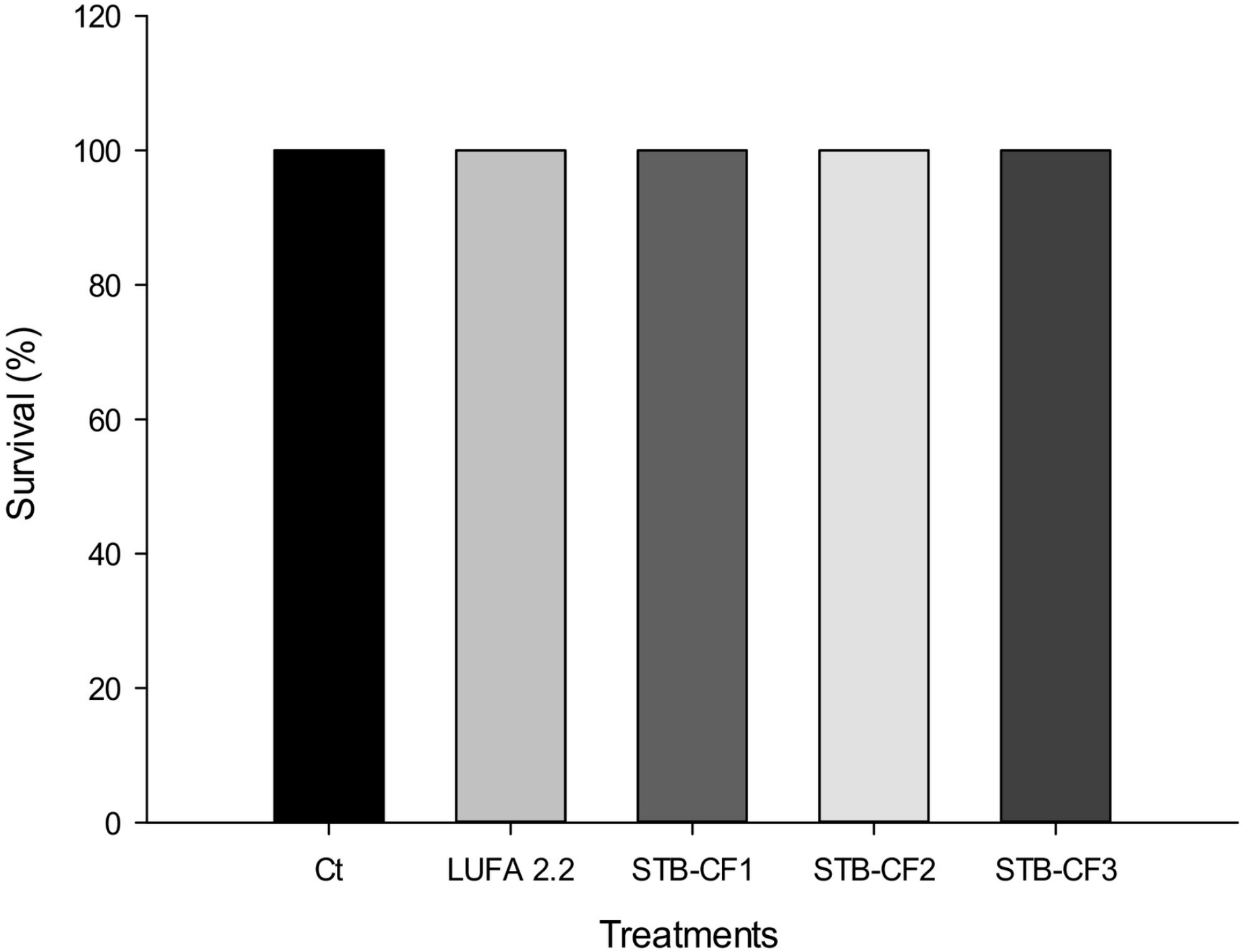
120
100
80
60
40
20
0
Survival (%)
Ct
LUFA 2.2
STB-CF1
STB-CF2
STB-CF3
Treatments

Figure 3

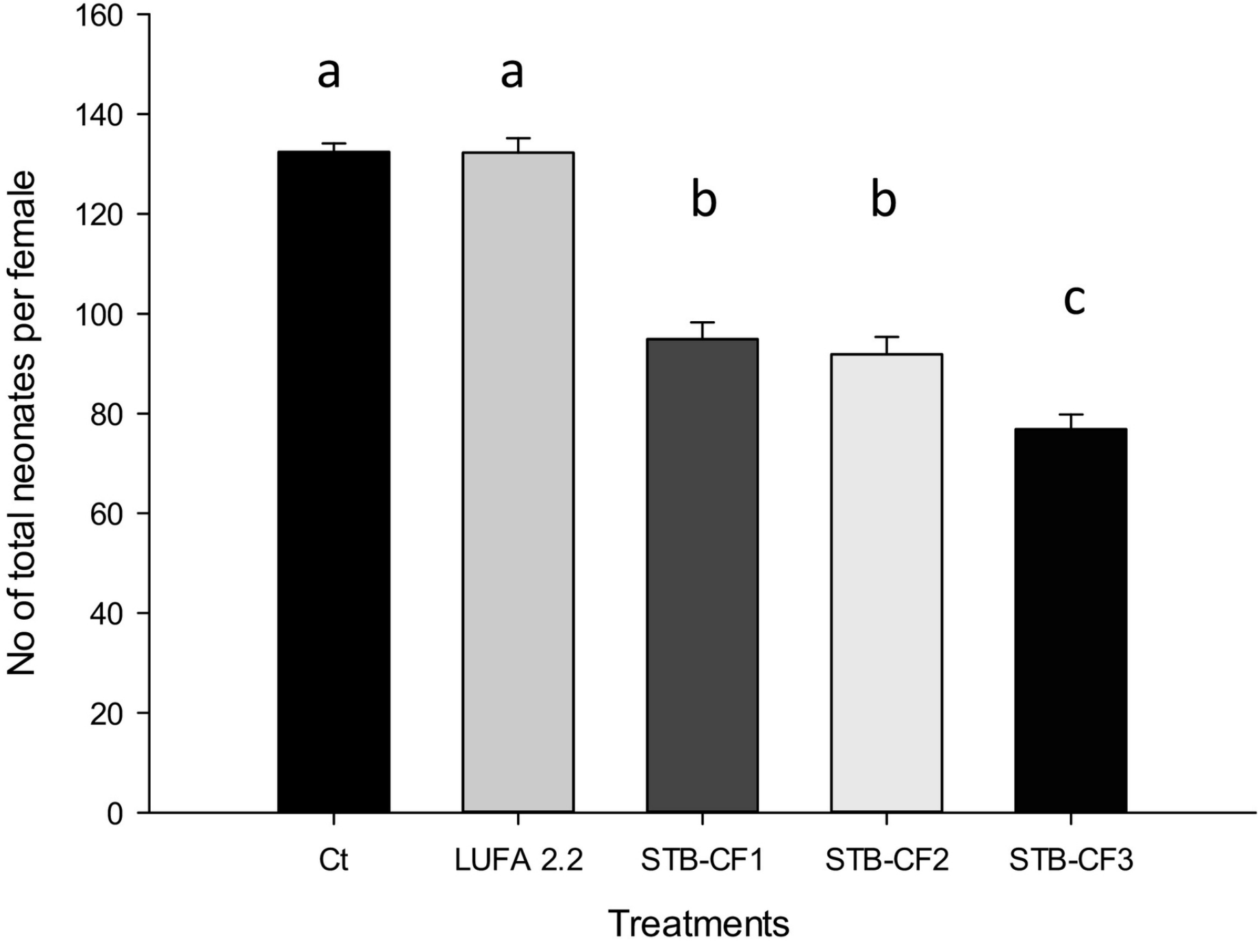
160
140
120
100
80
60
40
20
0
No of total neonates per female
a
a
b
b
c
Ct
LUFA 2.2
STB-CF1
STB-CF2
STB-CF3
Treatments

Figure 4

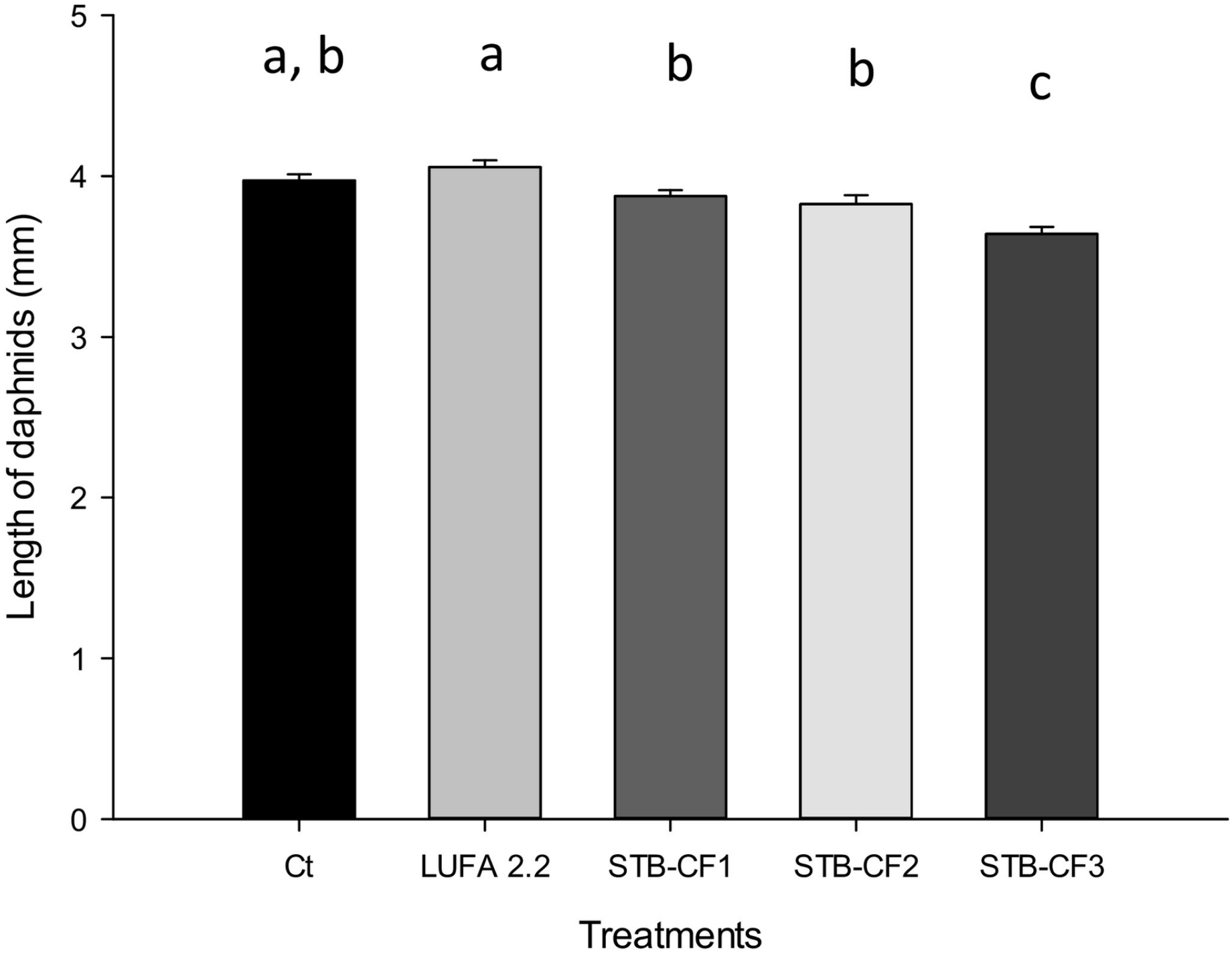
5
4
3
2
1
0
a, b
a
b
b
c
Length of daphnids (mm)
Ct
LUFA 2.2
STB-CF1
STB-CF2
STB-CF3
Treatments

Figure 5

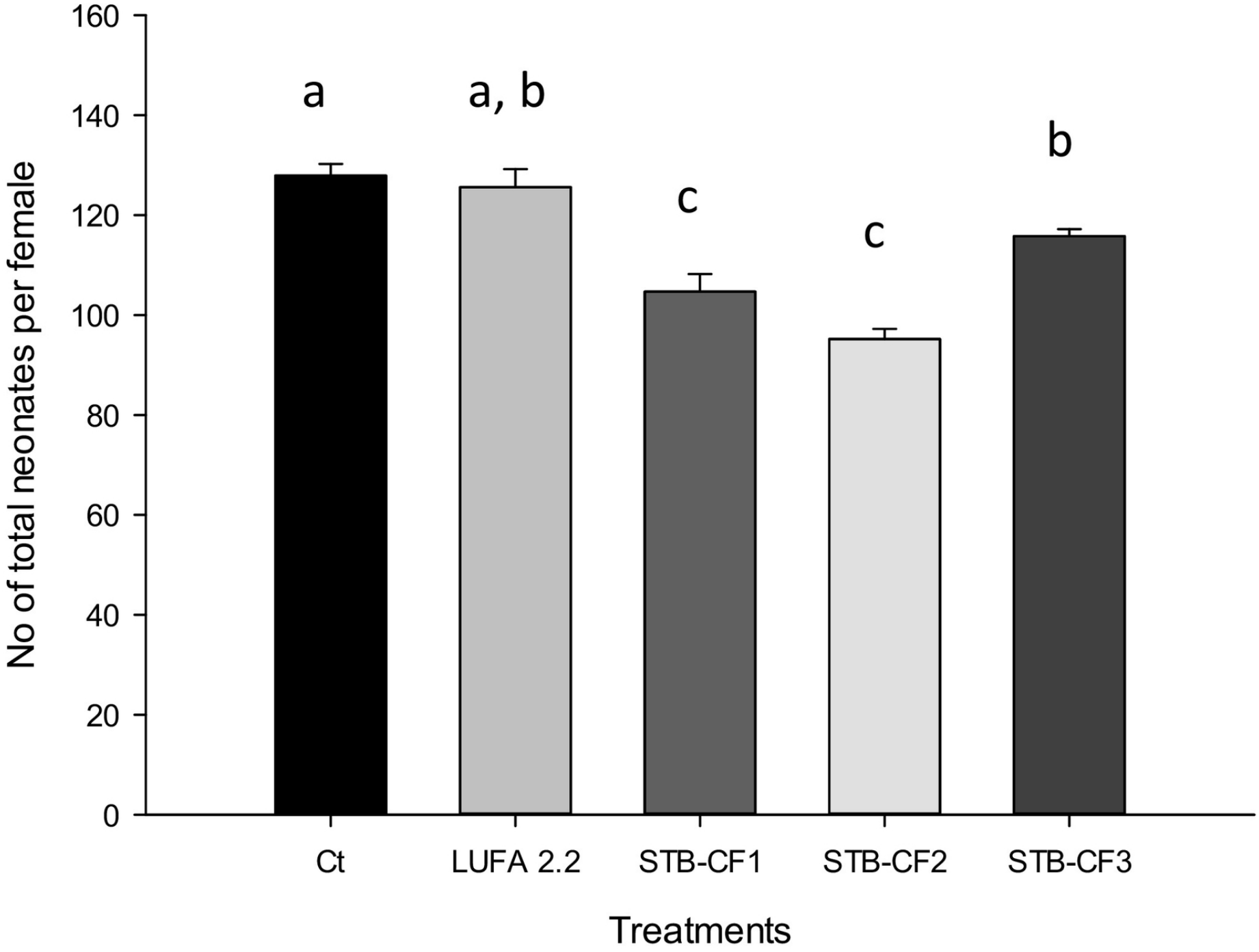
160
140
120
100
80
60
40
20
0
No of total neonates per female
a
a, b
c
c
b
Ct
LUFA 2.2
STB-CF1
STB-CF2
STB-CF3
Treatments

Figure 6

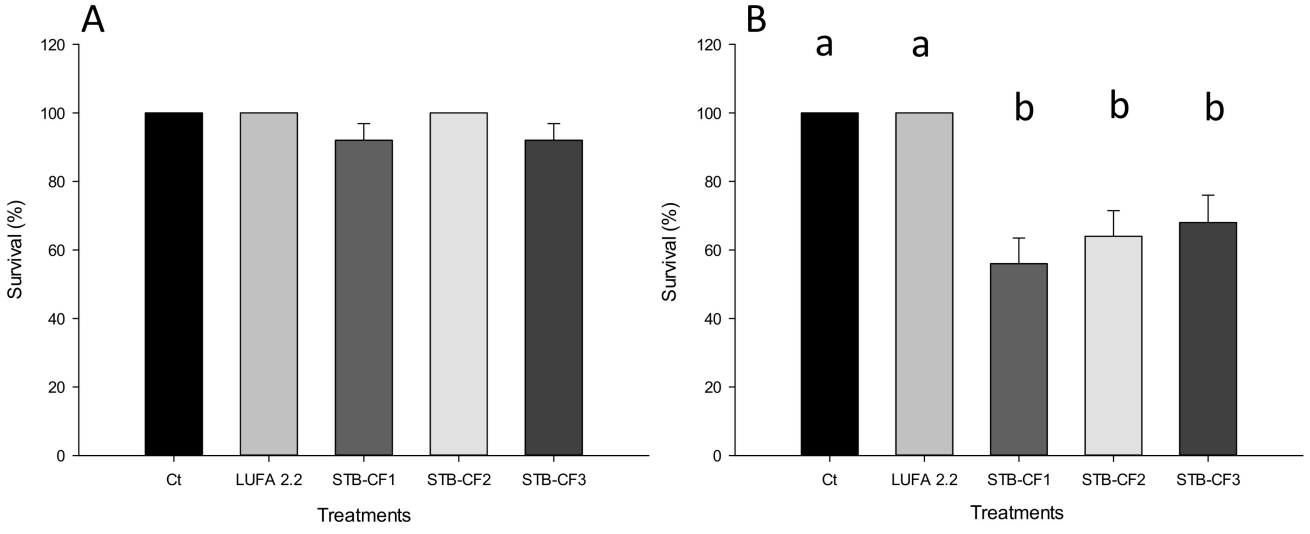
A
B
Survival (%)
Treatments
Ct
LUFA 2.2
STB-CF1
STB-CF2
STB-CF3
a
a
b
b
b
0
20
40
60
80
100
120

Figure 7