OCSC 499B: Thesis

Anesthesia in echinoderms: an experimental study of efficacy based on behavioural and cellular stress responses

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Abstract

While invertebrates make up most of the biodiversity on Earth, they remain understudied and have received limited attention relative to vertebrates when it comes to the complex issues of anesthesia. There is a knowledge gap that exists about whether and how invertebrates feel emotions or pain, therefore, animal care committees are often ambiguous on the need for anesthetics while performing experimental procedures on invertebrate taxa. Most of the anesthetics currently used for invertebrates have been adapted from protocols developed for vertebrates, under the unverified assumption that they are effective in blocking pain and/or reducing stress. For this reason, the focal species chosen for this study was a member of Echinodermata, the most closely related invertebrate phylum to vertebrates. In this study, four anesthetics that are currently used in the literature were tested for their efficacy, based on behavioural and cellular responses used to quantify stress levels in *Cucumaria frondosa*. The anesthetic agents that were tested include: ethanol, clove oil, MS-222 and $MgCl_2$. The behavioural metrics included reaction to a physical stimulus after anesthesia to test for immobilization, and measurements of cloacal respiration rate at the beginning and end of the anesthetizing procedure to test for a stress response. The cellular metrics included terminal counts and measurements of coelomocytes, which are known to spike during perceived threats in the focal species. Ethanol was not effective as an anesthetic as it evoked behavioural and cellular stress responses. $MgCl_2$ and clove oil were promising anesthetic agents but responses suggest that higher concentrations or longer exposures may be required. MS-222 seemed to have the most promise but was perhaps too harsh/potent based on the ambiguous behavioural response. This work highlights the complexity in finding effective invertebrate anesthetics, and that more research is needed to confirm the efficacy of the chemicals tested and explore alternate ones.

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List of definitions, abbreviations, and symbols

Abbreviation	*Meaning*
Ethanol	C_2H_5OH
Clove oil	Eugenol, $C_{10}H_{12}O_2$, solution made at a 1:9 ratio with 100% ethanol
$MgCl_2$	Magnesium chloride
MS-222	Tricaine methanesulfonate, $C_{10}H_{15}NO_5S$
Cloacal respiration	Each time a sea cucumber opened and closed its cloaca, it was considered one respiration
t_0	0-5 min period after immersion in anesthetic or control bath
t_1	15-20 min period after immersion in anesthetic or control bath

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1. Introduction

An anesthetic is a chemical that induces the loss of consciousness or sensation, generally used to prevent an organism from consciously feeling pain (Bloom and Cuthbert, 2018). While the purpose of anesthesia is apparent in humans and many other members of the Chordata phylum (vertebrates), it remains ambiguous for nearly all other animals (invertebrates), due to the differences in their nervous systems. In humans, anesthetics are used for surgeries, to provide lifesaving procedures that would be unbearably painful without them. In other vertebrates, such as fish, anesthetics are used for humanely carrying out dissections, examinations, handling, and more, in a manner that limits pain and reduces stress. In contrast, much less is known about the necessity and suitability of anesthetics for invertebrates, even though this group makes up most of the animal diversity on the planet (Cooper, 2001; Cooper, 2011; Wahltinez et al., 2022) where they play invaluable roles (e.g. as ecosystem engineers, nutrient cyclers and bioturbators). In addition, their study can lead to practical applications, such as aquaculture and the development of nutraceuticals (Cooper, 2001). There is an ongoing debate in the scientific community about whether (and how) invertebrates feel emotions or pain, leaving long-standing questions about the use of anesthetics with most taxa (Cooper 2001; Cooper, 2011). The use of anesthetics in marine invertebrates has been reported in species of Mollusca (Cephalopoda and Gastropoda), Arthropoda (Crustacea), Echinodermata, and few other phyla (Table 1 in Lewbart et al., 2012). The efficacy of these anesthetic agents has not yet been tested in conjunction with stress levels, with most of the current studies tending to assume that if the animal seems relaxed (limp/unresponsive), it is anesthetized. Due to the ongoing ambiguity about the need to alleviate pain and stress during experimental procedures with invertebrates, they are generally not subject to formal animal care protocols, with the possible exception of cephalopods (Smith, 1991).

Some scientists still choose to use routine anesthesia during procedures with marine invertebrates, relying on agents that have been adapted from knowledge gathered with fish, such as tricaine methanesulfonate (MS-222, $C_{10}H_{15}NO_5S$) and eugenol (clove oil, $C_{10}H_{12}O_2$) (Javahery et al., 2012; Lewbart et al., 2012), without confirming the mode of action of known anesthetics in invertebrate systems. This poses the question, do these anesthetics used in current practices truly work for marine invertebrates? Or do they cause more stress overall, which would make the entire process counterproductive and potentially worse? There are some anesthetics that have been used in invertebrates for some years, such as magnesium chloride ($MgCl_2$) and ethanol in molluscs (Lewbart et al., 2012; Abbo et al., 2021). Magnesium chloride has also been used in echinoderms and is the preferred method for anesthesia and euthanasia (Harms, 2006). Although their use in invertebrate anesthesia is much more prevalent and supported than the anesthetics adapted from vertebrates, there is still cause to test for a stress response, to verify that these anesthetics are working as well as they have been assumed to be for years.

Many gaps in knowledge raise uncertainty when it comes to addressing the question of invertebrate anesthesia. While the loss of consciousness (e.g. brain activity, ventilation, eye movements) can be measured in vertebrates such as fish to determine if they are conscious and feeling any sort of pain (Bowman et al., 2020), the signs are not obvious in most invertebrate taxa, especially those with no cephalic region, and no central nervous system. One role of an anesthetic is to render the subject immobile; however, it additionally needs to do so in a humane fashion and ultimately block all sensations, including pain and stress. While some marine invertebrates may appear anesthetized at first glance (limp/unresponsive), they could still be exhibiting forms of distress that go unnoticed until a proper way is devised to measure such reactions at the physiological, cellular or hormonal levels.

This project seeks to start answering some of the key questions surrounding the use of anesthesia in marine invertebrates, using behavioural and cellular metrics for stress. The model organism chosen is the sea cucumber *Cucumaria frondosa*, a member of the phylum Echinodermata, and the class Holothuroidea. Using the invertebrate phylum that is the most closely related to phylum Chordata (vertebrates), inside the deuterostome clade, makes it relevant for a preliminary assessment of the efficacy of common anesthetics. Another reason why *C. frondosa* is a worthy focal species is because it has recently been shown to exhibit measurable cellular and hormonal responses (spike in coelomocytes and cortisol) to perceived threats, which is akin to stress/anxiety (Caulier et al., 2020; Hamel et al., 2021). Practically speaking, their uniquely large Polian vesicle acts as an easily accessible reserve of coelomocytes that can be used in standard stress assays (Jobson et al., 2021).

Four anesthetic agents and protocols described in the current literature were tested to see if they work to immobilize the subject, and if so, whether the procedure causes any stress, to provide the first evidence in determining whether it would be more ethical to use the anesthetics or not. Agents were selected based on their common use with marine taxa, including ethanol (C_2H_5OH), magnesium chloride ($MgCl_2$), tricaine methanesulfonate (MS-222, $C_{10}H_{15}NO_5S$) and clove oil (Eugenol, $C_{10}H_{12}O_2$). It was hypothesized that if the anesthetic agent works, the individuals will become numb (e.g. non-responsive to poking) following anesthesia (20 min) and will be able to fully recover from the procedure. They must have a reduced respiration rate during immersion in anesthesia. In addition, non-lethal and non-stressful anesthetics must not cause a spike in the cellular responses of the focal species during or immediately after the procedure. This study hopes to reveal whether certain anesthetic agents work better than others, and which cause the least amount of behavioural and cellular stresses. Ultimately,

recommendations for animal care protocols could be made, by identifying the most ethical anesthetics for echinoderms and possibly other marine invertebrates.

2. Methods

2.1 Experimental design

Sea cucumbers collected from nearby locations that had been acclimated to flow-through conditions in the laboratory over several weeks were used. The individuals were kept in large tanks with their conspecifics. The study individuals were selected (n=48; weighing 50-150 g eviscerated wet weight) and placed into a smaller holding tank with the same flow-through conditions on the day before the trial. At the onset of the trial, they were placed individually into either a treatment or control beaker (5 L). The beakers were filled with seawater directly pumped from Logy Bay; temperatures ranged from 4.2 to 15.9 °C, depending on the dates of the trial. The beakers had nothing in them except for the seawater (with or without the anesthetic), and the sea cucumber. A total of 6 individuals (3 treatments and 3 controls) were used in each of two trials, such that a total of 6 treatment individuals and 6 control individuals were obtained for each chemical agent tested. 20-minute experiments were performed across a period from 9:00 AM to 4:00 PM, and were completed within a few weeks to avoid potential biases from known diurnal and seasonal cycles of *Cucumaria frondosa* (Gianasi et al., 2020). The first experiment of the day was always a control, followed by treatment experiment, then this order was repeated twice more so that 6 experiments were completed in one day, alternating between exposures. The light intensity was always below 58 lux, with an average across all the trials of 32 lux.

2.2 Experimental procedures

2.2.1 Anesthetic agents

The anesthetic agents tested are shown in Table 1; stock solutions, where necessary, were prepared on the eve or morning of the corresponding trials and refrigerated until use. Ethanol (5%) was made by diluting 100% ethanol directly in the beaker of natural seawater to the appropriate concentration and stirring. To make the 0.125 mL L^{-1} clove oil solution, it was first diluted in a 1:9 ratio in 100% ethanol because clove oil is poorly soluble in water; this stock solution was added to the beaker to get the desired concentration and then stirred. To make a 0.8 g L^{-1} solution of MS-222, the desired amount of powder was added to the seawater and stirred. For $MgCl_2$, a 1 M stock solution was made with filtered seawater (100 µm); this was added to the beaker at a 1:1 ratio with natural seawater to reach the desired concentration (7.5%), then stirred. References for each chemical concentration can be found in Table 1. Each chemical solution was made the same way across the treatment trials.

2.2.2 Exposure to anesthetic agents

The purpose of this experiment was to assess whether the anesthetic agents caused behavioural and cellular responses using an exposure time of 20 minutes, which was chosen to standardize the trials and to broadly align with the current literature (noting that variation occurs depending on the anesthetic; Table 1). The starting times for each replicate individual/beaker in a trial were staggered to allow for experimental procedures and samplings to be completed. The order of the experiments was always switching between control and treatments to minimize bias due to time of day. Behavioural metrics including cloacal respiration were measured at the beginning and end (t_0 = 0 min, t_1 = 15 min) for 5 minutes each time (see details in 2.3.2). After 20 minutes of exposure, each individual was briefly poked with a metal probe and its reaction

was scored (see details in 2.3.1). All treatment and control individuals were thereafter terminally sampled. The individuals were dissected to drain the fluid from the Polian vesicle where subsamples of fluid were collected for coelomocyte analysis (see details in 2.3.3). Fluid was set aside in a -80 °C freezer for later cortisol analysis, based on procedures developed by Jobson et al. (2021). The coelomocytes were placed on a hemocytometer analyzed under a microscope (Nikon Eclipse 80i) in a timely fashion to avoid cell aggregation (see details in 2.3.3).

2.2.3 Recovery from anesthesia

In order to confirm that the anesthetizing procedure was not permanently damaging or lethal, a series of trials used the same protocols as above, except the subjects were not terminally analyzed. Instead, after the trial, the control individuals and those exposed to the anesthetic agent were returned to individual recovery vessels (5 L beakers with flowing seawater). Their condition was assessed immediately post transfer, again after 24 h, and finally after one week. Metrics measured at set time intervals (t_0 = 0 min, t_1 = 15 min, t_2 = 24 ± 2 h, t_3 = 1 week) included cloacal respiration and poking the individuals 3 times with a metal probe to score the physical reaction (t_0 excluded from the poking) (Table 2). Additional metrics such as attachment to the substrate and deployment of the feeding tentacles were used to determine whether the sea cucumbers were acting ‘normal’.

2.3 Metrics recorded

Two behavioural metrics were used; one was the physical response to a stimulus to test for immobilization, the other was cloacal response as a proxy of stress level. A cellular metric was also used to measure the stress response.

2.3.1 Physical response

This behavioural metric provided information on the general efficacy of the procedure at rendering the subject unresponsive. After the 20-minute exposure to either seawater (control) or seawater with desired anesthetic concentration (treatment), the individuals were exposed to a physical stimulus. This involved poking the sea cucumber in the middle of its body with a metal probe three times, each poke with the same applied pressure. The response to this stimulus was then observed and scored on a scale of 0 (unresponsive) to 3 (rapid contraction, considered the normal reaction of a healthy sea cucumber) (Table 2).

2.3.2 Cloacal respiration

This behavioural metric was used to evaluate stress as in previous studies (Gianasi et al., 2020; Jobson et al., 2021). Cloacal respiration in sea cucumber is defined as the opening and closing of the cloaca (sometimes referred to as the anus), to pump seawater in and out of the respiratory tree where oxygenation occurs (Jaeckle & Strathmann, 2013).

Each time the cloaca opened and closed, it was counted as one respiration. For each sea cucumber, counts were taken over two periods of 5 minutes, once at the beginning of the procedure (t_0) and once at the end (t_1). The total cloacal respiration counts for each time period were averaged to give cloacal respiration rates per min.

2.3.3 Coelomocytes

Coelomocytes are cells present in the coelomic fluids of sea cucumbers and other echinoderms (Jobson et al., 2021). It was recently demonstrated that coelomocytes spike in the presence of foreign particles (Caulier et al., 2020) or when sea cucumbers perceive potential threats (Hamel et al., 2021).

A small opening was made with a scalpel near the posterior end of the body, between rows of tube feet. Scissors were then used to cut the body open longitudinally towards the anterior end. This took approximately 5 minutes. Following a protocol developed by Caulier et al. (2020) and Jobson et al. (2021), the Polian vesicle was removed and isolated and its fluid poured into a petri dish, transferred to 15 mL vials, and the total amount noted this took approximately 5 minutes. Subsamples for cortisol analysis (planned for the future) were stored into 1.5 mL centrifuge tubes in a -80 °C freezer. All organs were then removed, and the eviscerated body weight (which included muscle bands and aquapharyngeal bulb) was measured and recorded.

Coelomocyte analysis began immediately after collection due to a tendency for them to agglomerate quickly, as mentioned by Caulier et al. (2020), and was concluded approx. 15-25 minutes after collection. During this time period, vials were swirled to keep the cells suspended. Aliquots of mixed fluid were placed into a hemocytometer using a pipette, then a cover slip was applied sequentially. Cells were examined under the microscope (Nikon Eclipse 80i) at 200x magnification; 4 photos were taken of each side of the hemocytometer, for each sample of fluid, yielding a total of 8 photographic samples per individual. The squares chosen for the photos were randomly generated from 1-25, in order to minimize any bias. Each photo was analyzed for number and types of coelomocytes. There were four coelomocyte types recorded and monitored: phagocytes, morula, crystal, and fusiform (Figure 1). Aggregates of coelomocytes, defined as any cluster of ≥ 3 cells, were also identified and counted (Figure 1). Free coelomocytes and aggregates were categorized and analyzed separately but added together for total coelomocyte analysis.

2.4 Data analysis

All of the statistical analyses were performed in JASP and used $\alpha=0.05$ for significance. Assumptions of normality (Shapiro-Wilk for t-tests, Q-Q plot of residuals for ANOVA) and equal variance (Levene's test) were first explored to confirm the use of parametric tests. If either of the assumptions failed, the data was log transformed and tested again. If this failed to correct data distributions, a non-parametric test was conducted.

A Mann-Whitney U test was used to test the differences between behavioural scores in the treatment versus control groups for each chemical agent. A two-way analysis of variance (ANOVA) was first performed to test the differences between the cloacal respiration rates of control and treatment groups at t_0 and t_1. Because of a significant interaction between treatment and time within the clove oil trial, independent one-way ANOVA tests had to be conducted for each of these factors.

The means of total coelomocyte density for control and treatment groups of each chemical was analyzed with a t-test. The data for $MgCl_2$ and MS-222 had to be log transformed due to failure of the normality test. Once transformed, both data sets passed the assumption test. Similarly, t-tests were performed for comparison between control and treatment group means for free coelomocyte density and for phagocyte density.

3. Results

3.1 Behavioural responses

3.1.1 Response to a physical stimulus

Figure 2 shows graphical results of exposure to the treatments and Table 3 lists the corresponding statistical results. Exposure to MS-222, clove oil, and $MgCl_2$ visibly decreased the

response to a physical stimulus in comparison with their respective control groups. MS-222 was the most successful chemical, with individuals submitted to this treatment not showing any observable movement at all (0±0, n=6), in contrast to the corresponding movements elicited in the control group (1.5±1.2, n=6). Clove oil did not fully eliminate the response as there was still movement from the treatment individuals (0.83±0.75, n=6) but it was the second most effective at significantly reducing the response compared to its control (1.8±0.75, n=6). Individuals exposed to $MgCl_2$ showed a mean response that was lower (1.3±0.52, n=6) but not significantly different from that of the control group (1.7±0.8, n=6). Inversely, individuals exposed to ethanol showed a higher behavioural score (1.5±1.0, n=6) compared to their controls (0.83±0.75, n=6), but the difference was not significant.

3.1.2 Cloacal respiration

Figure 3 represents the cloacal respiration rates for each of the treatment and control groups within each of the chemicals at two different time intervals, and Table 4 provides the statistical results. Overall, the effect of treatment was only significant for clove oil and MS-222, and the effect of time was not significant for any treatment. There was one instance of a significant interaction of treatment and time, this was in clove oil.

At t_0, the ethanol treatment elicited a cloacal respiration rate that was higher (2.4±0.57, n=6) than the control group (1.9±0.75, n=6), but not significantly so. Similarly for t_1, ethanol induced a non-significantly higher (2.9±0.93, n=6) cloacal respiration rate when compared to the control (2.4±1.1, n=6). $MgCl_2$-treated individuals showed a cloacal respiration at t_0 (2.1±0.64, n=6) that was similar to the control group (2.1±0.33, n=6). By t_1, the treatment group had a lower cloacal respiration rate (1.9±0.47, n=6) than the control (2.5±1.3, n=6), but not significantly so.

In contrast, the cloacal respiration rate of individuals was significantly decreased/blocked by the presence of MS-222. The treatment group had an initial cloacal respiration rate (0.10±0.17, n=6) that was significantly lower in comparison to the control (1.9±0.79, n=6). The trend was maintained for the second time interval, showing again that the treatment caused an almost complete cessation (0.083±0.16, n=6) of cloacal respiration, compared to the control group (2.7±0.55, n=6). Finally, analysis of the clove oil results showed a significant difference between treatment (1.9±0.47, n=6) and control (2.1±0.78, n=6) at t_0, as well as at t_1 (treatment: 1.1±0.95, n=6; control: 2.6±0.59, n=6). Clove oil also showed a significant interaction between treatment and time, which means that the treatment of clove oil decreased the respiration rate over the time submerged in the anesthetic. These results were then analyzed separately using a one-way ANOVA, and the significance did not change (Table 4).

3.2 Cellular response

3.2.1 Total coelomocytes

This section summarises the results for pooled coelomocyte types (free coelomocytes and aggregated coelomocytes) and the statistical analysis can be found in Table 5. It should be noted that all the coelomocyte densities are in millions of cells (x 10^6) per mL. At the end of the exposure, coelomocyte densities in the ethanol (11.8±7.4, n=6) and MS-222 (16.7±15.6, n=6) treatments presented higher mean values than their respective controls (8.63±4.8, n=6; 14.8±5.7, n=6) which can be seen in Figure 4A and C. However, these differences were not significant. Coelomocyte densities in individuals exposed to ethanol were 37.2% higher than their respective control group and those in the MS-222 treatment were 12.9% higher than their control. Individuals exposed to clove oil displayed a coelomocyte density (10.5±6.3, n=6) that was only 1.6% higher than the control group (10.3±6.9, n=6) which was not a significant difference

(Figure 4B). Finally, individuals exposed to $MgCl_2$ displayed a 16.2% lower average coelomocyte density (11.8±9.2, n=6) when compared to the control (14.1±5.2, n=6) (Figure 4D), without a statistically significant difference.

3.2.2 Free coelomocytes

Free coelomocytes can be sorted into cell types: phagocyte, morula, crystal, and fusiform (Figure 1). The free coelomocyte densities are represented independently in Figure 5 to have a closer look when aggregates are not dominating the histogram. It should be noted that the coelomocyte densities are shown as x 10^5 cells per mL. Sea cucumbers exposed to ethanol showed a similar result for free coelomocytes as for the previously outlined total coelomocytes, with a 62% higher density in the treatment (16.3±9.3, n=6) than the control (10.1±4.1, n=6). The exposure to clove oil also showed comparable results, with free coelomocyte densities in the treatment (9.4±5.5, n=6) having only a 4.9% lower density than the control (9.8±4.4, n=6). MS-222 produced a clearer response with free than total coelomocytes; individuals submitted to this treatment had free coelomocytes densities (9.6±4.3, n=6) that were 23.9% lower than the control (12.7±3.4, n=6). The free coelomocyte results after exposure to $MgCl_2$ were comparable to the total coelomocyte densities, with an 8.4% lower density in treatment (12.5±8.5, n=6) than control (13.6±4.9, n=6). All of these differences remained non-significant (Table 6).

3.2.3 Free coelomocytes deconstructed

The free coelomocytes subcategories were further explored (Figures 6 and 7) using values that are x 10^5 cells per mL for phagocytes, and x 10^4 cells per mL for the remaining coelomocyte types. Phagocyte densities (Figure 6) in the ethanol treatment group (14.8±8.9, n=6) were 66.1% higher than in the control (8.9±3.7, n=6). Sea cucumbers exposed to $MgCl_2$ and MS-

222 showed lower values than the control densities, the $MgCl_2$ treatment group (12.1±8.2, n=6) was 4.1% lower than its control (12.6±4.1, n=6) and the MS-222 treatment group (12.1±4.7, n=6) 2.5% lower than its control (12.4±2.8, n=6). In an interesting contrast, phagocyte density in the clove oil treatment group (12.0±2.4, n=6) was 29.9% higher than in the control group (9.2±4.5, n=6). However, there were no significant differences between phagocyte densities in treatment versus control groups for any of the chemicals tested (Table 6).

The less abundant morula, crystal and fusiform cells are shown in Figure 7. Crystal cells were the least abundant coelomocytes; they were found in all sea cucumbers, except for the $MgCl_2$ treatment group. Densities of morula and fusiform cells were higher than that of crystal cells, but still not comparable to phagocyte abundance. These numbers were so sparse that they most likely did not make a difference in coelomocyte densities.

4. Discussion

4.1 Preamble

This project aimed to start exploring the complexity of invertebrate anesthesia. Until now, there had been few studies that had evaluated the efficacy of anesthetics used to immobilize and desensitize marine invertebrates (Lewbart et al., 2012), and none that tested the efficacy of anesthetics in conjunction with the individual's stress levels.

For an animal to be anesthetized, it must be immobilized, but this alone does not guarantee that the process is stress-free or painless. An example of this in vertebrates would be muscle paralysis in humans, while still maintaining perceptions and sensations that can generate stress. So for an anesthetic to be effective, it must not only immobilize but also block sensations and limit stress in the individual.

Determining senselessness in many invertebrate taxa is a challenge. The present study explored this notion using a combination of behavioural and cellular metrics. While findings provide preliminary guidance, it remains difficult to declare an anesthetic as completely effective. The ideal scenario would be all of the metrics falling under the preferred categories, such that physical response/movement is prevented, and both behavioural and cellular metrics are consistent with no or minimal stress. There is also the added element of recovery from the whole procedure, which would confirm the anesthetic to be both effective and non-lethal/detrimental (this study seeks anesthesia, not euthanasia).

4.2 Discussion of anesthetic agent efficacy

4.2.1 Immobilization

This metric answers the first question of the study: does the anesthetic cause the individuals to be unresponsive after the period of anesthesia? The sea cucumbers that were submerged in ethanol immediately started to move around, noticeably more than the individuals in the control group did. Ethanol also triggered the sea cucumbers to be more responsive compared to the control group when poked and scored for their physical response, indicating that this anesthetic was not effective and is unlikely to deserve further investigation. Clove oil and $MgCl_2$ both showed results that reduced the response to the physical stimulus after anesthesia, compared to controls. However, there was still a response, suggesting that the two anesthetics may have started to work but did not completely immobilize the sea cucumbers. Clove oil and $MgCl_2$ will need further investigation especially since the behavioural test was not designed with the intent to perform statistical analysis, but more so to observe the animal's behaviour and answer the clear question of efficacy. Higher concentrations or longer exposures could be explored, especially given the results of the stress metrics (discussed below).

One anesthetic stood out amongst the rest in the ability to immobilize, and that was MS-222. Once the treatment individuals were placed in this anesthetic bath, they became immobilized almost instantly. The behaviour scores in response to the physical stimulus was always 0 (no movement at all) in the treatment while the control individuals remained responsive. This anesthetic therefore shows the most promise for the next metrics of the study, which are the stress tests.

4.2.2 Cloacal respiration as a metric for stress

Cloacal respiration rates illustrate a non-invasive, observable stress metric of sea cucumbers (Gianasi et al., 2020; Jobson et al., 2021). The ideal cloacal respiration response of a sea cucumber would be a reduction in respiration rate; the adverse response would be a faster respiration rate. In relation to the time submerged in the anesthetic, it is ideal to have a relationship that decreases the cloacal respiration over time, as opposed to increasing it.

Unsurprisingly, the ethanol-treated group showed a higher cloacal respiration rate than the control group at the beginning and the end of the immersion period. This confirms that ethanol was not an effective anesthetic. $MgCl_2$ treatment elicited lower cloacal respiration than the control and a slight further reduction over time, suggesting that this chemical did not induce a clear stress response. The clove oil treatment group showed an interesting result during the period of immersion in the anesthetic. Cloacal respiration rates at the beginning and the end of the immersion period were both lower than the control groups, which is a positive sign. Given the significant interaction between treatment and time, clove oil clearly reduced the respiration rate over time. This is a promising result, confirming clove oil as a possible candidate for further study.

MS-222 once again stood out among the rest. Treatment individuals closed their cloaca upon entering the anesthetic bath, with very few displaying one last respiration after that. In a study done by Jobson et al. (2021), the cloaca of *C. frondosa* would close upon exposure to air. Here, in a similar fashion, the animals may have been trying to prevent the MS-222 from entering their systems through the respiratory tree. It is possible that this anesthetic was so potent, that the sea cucumber's defence was to freeze and close up its openings. There is also a possibility that the sea cucumber immediately lost the muscle strength to open and close its cloaca. Either way, the apparent potency of this anesthetic is promising, especially if the absence of cellular stress can be confirmed (discussed below).

To summarize the behavioural results, ethanol can be dismissed as an effective anesthetic. $MgCl_2$ is still up for debate and clove oil shows more promise after this test of cloacal respiration over time. Overall, MS-222 emerges as the anesthetic with the most dramatic (but still ambiguous) results.

4.2.3 Coelomocyte density as a metric for stress

Coelomocytes are versatile cells in echinoderms. Their main function relates to the immune system (Smith et al., 2010; Jobson et al., 2021), which is simple in comparison to vertebrates, being entirely based on an innate immunity, which is fast acting and non-specific (Jobson et al., 2022). Caulier et al. (2020) proved that coelomocytes spike in the presence of foreign particles, which is indicative of an immune response. If an anesthetic is triggering an immune response, it most likely means that the individual is still capable of feeling stress. In fact, spikes in coelomocytes have been shown to occur as a stress response to nearby predators (scent only) and injured conspecific (alarm signals) in *C. frondosa* (Hamel et al., 2021). In the Hamel et al. (2021) study, spikes in coelomocytes occurred and were monitored across a 180-

minute period, including at 30 minutes. The coelomocytes were sampled after 20 minutes in the present study.

The ethanol treatment group showed a spike when compared to the controls, confirming that cellular stress mirrored the adverse behavioural responses discussed above for this chemical, providing support for the methodology used to evaluate stress. Ethanol is not a natural chemical found in a marine environment; therefore, much like the Caulier et al. (2020) study, ethanol could elicit an immune response, or it can be perceived as a threatening chemical cue like in the study by Hamel et al. (2021).

MS-222 is a promising anesthetic agent when considering the behavioural responses, but the question is, do the cellular responses match? The total coelomocyte densities of the treatment group were not consistent with the absolute absence of stress. MS-222-treated individuals showed higher coelomocyte densities than their respective controls. Given the immediate closure of the cloaca, MS-222 could be perceived as a foreign substance to the sea cucumber and cause stress. The cellular response to clove oil, on the other hand, did not differ much from that of the control group, suggesting that while it did not render the sea cucumber limp, it did not trigger any cellular stress response either. Similarly, $MgCl_2$ elicited lower total coelomocyte densities in comparison to the control, which suggests it was relaxing the individuals despite not immobilizing them, perhaps because it is naturally found at low concentrations in seawater (Abdel-Aal et al., 2017).

It was evident in the fluid samples that aggregated coelomocytes largely outnumbered the free coelomocytes. Cellular clotting of immune cells is described in Smith et al. (2010) as a mechanism to respond to tissue damage and block coelomic fluid from escaping the body, and to also fight pathogens. Since it takes time for coelomocytes to agglomerate, and the exposure of

the sea cucumbers to each anesthetic was so short-lived, focusing on the freely suspended coelomocytes has the potential to show clearer results in conjunction with the behavioural metrics.

In this regard, the free vs total coelomocyte results were comparable for the ethanol, $MgCl_2$ and clove oil trials. However, MS-222 again stood out, with the free coelomocyte results showing a different scenario. There was a lower density of free coelomocytes within the MS-222-treated individuals compared to the controls, which aligns with the behavioural results better than the total coelomocytes. The difference in free coelomocytes and total (mostly aggregated) coelomocytes could possibly be from a sea cucumber that has been injured previously. As mentioned by Smith et al. (2010), coelomocytes tend to aggregate when there is tissue damage in an effort to minimize the loss of coelomic fluid. Perhaps a sea cucumber that was previously exposed to a tissue injury had residual aggregations of coelomocytes which altered these results. This cannot be confirmed, as the sea cucumbers used in this study were terminally sampled, but this would line up with all of the other results for MS-222. There was an aggregate in one of the MS-222-treated individuals that was estimated to be formed of 100 coelomocytes or more, which was the biggest aggregate found in this study, and seemed to be an outlier. This individual could have been an injured sea cucumber. The hormonal (cortisol) analysis that will be done later should help confirm whether the sea cucumbers were stressed by MS-222 or if the cellular aggregates introduced an inconsistency with the total coelomocyte counts.

Meanwhile, the free coelomocytes can be categorized into groups, with phagocytes being the most abundant by far. The main function of phagocytes is to engulf foreign particles and their abundance can be attributed to this role (Smith et al., 2010; Smith et al., 2018). For the ethanol trial, phagocyte results were consistent with all previous cellular results (more in the treatment

group than the control group). For clove oil, there were slightly more phagocytes in the treatment group, which differs from the free coelomocyte results. The $MgCl_2$ and MS-222 treatment groups both showed densities consistent with their respective controls. The explanation for these slight shifts in results could lie with the other coelomocyte types found in this study, which are not as plentiful as the phagocytes (Smith et al., 2018) and therefore do not hold as much weight. These cells all have unique functions. For example, morula cells have been shown to encapsulate bacteria, and can also form rims around wounds (Smith et al., 2010). The function of crystal cells remains ambiguous (Smith et al., 2018). The function of fusiform cells is also not well documented in the literature. Ethanol-treated individuals had the most free coelomocytes besides phagocytes, perhaps because this chemical triggered the most stress out of all the tested anesthetics. As for the other chemical trials, nothing clear emerged about the morula, crystal, and fusiform coelomocyte types, except for exposure to $MgCl_2$ where treatment individuals had no morula or crystal cells. This could be because $MgCl_2$ was not perceived as a threat, being naturally found in seawater (Abdel-Aal et al., 2017). Ultimately, these additional three types of coelomocytes did not make a large contribution to the free coelomocyte densities, but over a longer induction period in the anesthetics, they could multiply and provide a clearer picture.

4.3 Summary of anesthesia efficacy and future recommendations

Combining all three metrics of response to a physical stimulus, cloacal respiration rate, and coelomocyte densities will ultimately paint the picture of which anesthetics worked the best overall, and why. A comprehensive summary of the results can be found in Table 7. Ethanol was the most stress-inducing chemical for the sea cucumbers across all of the metrics. In contrast to its prominent use in molluscs at various concentrations (Lewbart et al., 2012), my recommendation is that this chemical should not be used for anesthesia or euthanasia in future

studies. Combining the results of each metric for clove oil reveals an anesthetic that has promise upon making some alterations to the protocol, as it reduced the physical response, significantly reduced the respiration rate over time, and kept the cellular stress response at a normal level. I would recommend increasing the concentration of this chemical and increasing the exposure time in future studies. The physical response could be further reduced with a higher concentration, and the respiration rate might decrease even more with a longer immersion in the anesthetic. $MgCl_2$ was also a promising anesthetic and could be effective if the right concentration was used. It reduced the physical response, reduced the respiration rate and kept the cellular stress response low. Increasing the concentration of this chemical could block the physical response and minimize the cellular response further. Finally, MS-222 clearly was the most potent. It completely immobilized the individuals, it blocked respiration, and it kept the free coelomocytes at low levels. In future studies, I would recommend decreasing the concentration, which might produce even less of a cellular response. I chose to use the higher concentration of the two in the study by Applegate et al. (2016; 0.8 g L^{-1}); however, I think the lower concentration of 0.4 g L^{-1} would be worth exploring in holothuroids because it might be more effective both biologically and economically.

4.4 Limitations of the study

4.4.1 Experimental limitations

The sample size was small due to the short time frame in which these experiments had to be completed, with n=12 individuals for an entire chemical trial (n=6 treatment, n=6 control). Perhaps with larger sample sizes there would have been clearer statistical significance in the results. Another limitation of this study was fixed concentrations. Ideally, it would have been better to test multiple concentrations of each chemical, but because of the time restraint, this

could not be done. It would have been ideal to also test multiple immersion times, so that the effect of time could be considered with both behavioural and cellular metrics. It is possible that coelomocyte densities in this study were sampled at the beginning of their spike; it is unknown whether a long-term trial could have revealed a greater difference between control and treatment groups for any of the agents. Another limitation is human error. I was counting and classifying each cell and aggregate myself. But since I was the only one counting, this error would be small because it was consistent. Due to the nature of the behavioural response scoring system including 0 as a rank, and the MS-222 treatment individuals all scoring 0, statistical analysis could not be done on this trial. In the future, I would use a scale of 1-4 instead of 0-3.

4.4.2 Specimen limitations

There could be a bias associated with the unknown sea cucumber ages. Individuals of a consistent size were used but there was no way to tell their age besides determining that they were sexually mature. There is also the issue of each sea cucumber being a different organism (personality); in a behavioural study we must assume that combining enough individual reactions can account for an entire population. There was also the possible limitation of unknown previous injury. As mentioned in the discussion, when working with aggregated coelomocytes, there could be residual aggregates from previous tissue injuries.

4.5 Future work

The next step following this study will seek to confirm the efficacy of the most promising anesthetics. Anesthetizing the animals as described before, and placing them in a tank with their predator, *Solaster endeca*, will reveal whether the sea cucumbers are truly rendered senseless upon examining the same stress metrics; cloacal respiration rate and coelomocyte density (focusing now on free coelomocytes). In a study by Hamel et al. (2021), it was shown that sea

cucumbers exhibit a spike in coelomocytes when exposed to the scent of *S. endeca*. Trials of control and treatment (anesthetized) groups exposed to a predator will reveal whether an anesthetic like MS-222 truly anesthetizes the sea cucumbers, or if it just immobilizes them. If the anesthetic works, the stress levels of the treatment individuals should not increase, and the control individuals should show a stress response similar to what was seen in Hamel et al. (2021). If sea cucumbers do show a stress response to the predator, then the anesthetic is not 100% effective.

Tables

Table 1. Summary of anesthetic agents and concentrations tested on *Cucumaria frondosa*. Chemical agents include ethanol, eugenol (clove oil), tricaine methanesulfonate (MS-222), and magnesium chloride ($MgCl_2$).

Chemical	**Concentration**	**Reference**
Ethanol	5% of SW solution	Lewbart et al., 2012
Clove oil	0.125 mL L^{-1}	Lewbart et al., 2012
MS-222	0.8 g L^{-1}	Applegate et al., 2016
$MgCl_2$	7.5% 1:1 ratio with seawater	Lewbart et al., 2012; Pugliese et al., 2016

Table 2. Scale of responses exhibited by individuals of *Cucumaria frondosa* to a physical stimulus consisting of a metal probe poking them three times after 20-minute exposure to anesthetizing agent.

Response	Score
No movement	0
Slow body wall contraction (> 3 seconds)	1
Fast body wall contraction (< 3 seconds)	2
Fast body wall contraction (< 3 seconds) + tentacles retraction	3

Table 3. Statistical results for Mann-Whitney U test comparing the behavioural scores of control and treatment groups of each chemical agent.

Chemical agent	**W**	***p* value**
Ethanol	11.000	0.273
Clove oil	29.500	0.062
MS-222*	-	-
$MgCl_2$	22.000	0.523

*Did not test due to treatment scores being consistently zero.

Table 4. Statistical results of individual two-way analysis of variance (ANOVA) comparing cloacal respiration rates in the control and treatment groups at t_0 and t_1 for each anesthetic trial.

Ethanol			
Cases	**F statistic**	**df**	***p* value**
Exposure (Control/Treatment)	1.307	1	0.200
Time (t_0 and t_1)	1.307	1	0.200
Exposure and Time	0.000	1	1.000
Clove oil			
Cases	**F statistic**	**df**	***p* value**
Exposure (Control/Treatment)	9.346	1	0.006
Time (t_0 and t_1)	0.321	1	0.578
Exposure and Time	4.628	1	0.044
Clove oil – factors analyzed separately (one-way ANOVA)			
Cases	**F statistic**	**df**	***p* value**
Exposure (Control/Treatment)	8.242	1	0.009
Time (t_0 and t_1)	0.208	1	0.653
MS-222			
Cases	**F statistic**	**df**	***p* value**
Exposure (Control/Treatment)	120.537	1	<0.001
Time (t_0 and t_1)	3.174	1	0.090
Exposure and Time	3.476	1	0.077
$MgCl_2$			
Cases	**F statistic**	**df**	***p* value**
Exposure (Control/Treatment)	0.703	1	0.412
Time (t_0 and t_1)	0.099	1	0.757
Exposure and Time	0.889	1	0.357

*For clove oil, one-way ANOVA was used to analyze the effects of time and treatment independently due to a significant interaction term.

Table 5. Statistical results of t-test comparing total coelomocyte counts in control and treatment groups for each chemical agent.

Chemical agent	**t statistic**	**df**	***p* value**
Ethanol	-0.888	10	0.396
Clove oil	-0.042	10	0.967
MS-222*	0.273	10	0.790
$MgCl_2$*	1.009	10	0.337

*Data was log transformed due to failure of normality (Shapiro-Wilk) assumption.

Table 6. Statistical t-test results for comparisons between free coelomocyte density and phagocyte density in control and treatment groups for each chemical agent.

Free coelomocytes			
Chemical agent	**t statistic**	**df**	***p* value**
Ethanol	-1.502	10	0.164
Clove oil	0.167	10	0.871
MS-222	-0.235	10	0.819
$MgCl_2$	0.286	10	0.781
Phagocytes			
Chemical agent	**t statistic**	**df**	***p* value**
Ethanol	-1.496	10	0.166
Clove oil	-1.328	10	0.214
MS-222	-0.167	10	0.871
$MgCl_2$	0.139	10	0.892

Table 7. Summary of behavioural (response to a physical stimulus, cloacal respiration rate), cellular (coelomocyte densities) and recovery (survival) results exhibited by individuals of Cucumaria frondosa after exposure to anesthetic agents for 20 minutes.

Chemical	Physical stimulus	Cloacal respiration	Total coelomocyte response	Free coelomocyte response	Recovery
Ethanol	Control < Treatment	Control < Treatment	Control < Treatment	Control < Treatment	Survived
Clove oil	Control > Treatment	Control > Treatment	Control = Treatment	Control = Treatment	Survived
MS-222	Control > Treatment	Control > Treatment	Control < Treatment	Control > Treatment	Survived
$MgCl_2$	Control > Treatment	Control > Treatment	Control > Treatment	Control > Treatment	Survived

*Note that these trends may not all be statistically significant; see Tables 3, 4, 5 , 6 for statistical analysis.

Figures

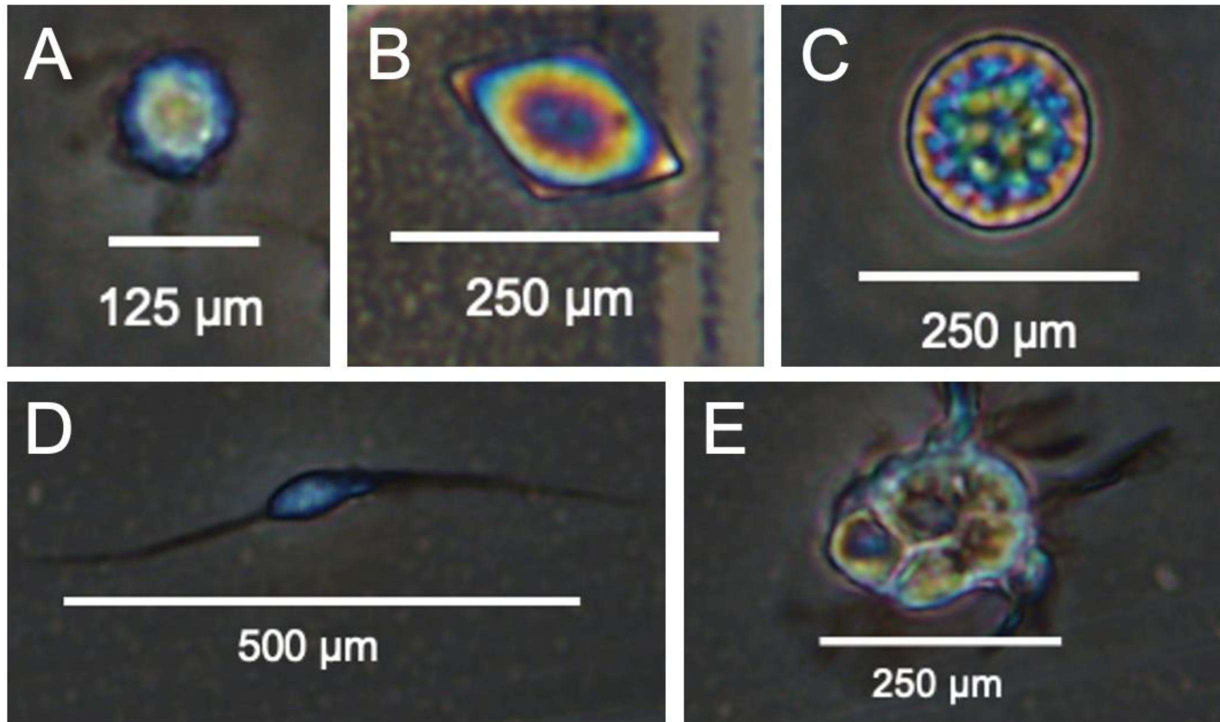

Figure 1. The coelomocyte types of the sea cucumber C. frondosa under 200X magnification on Nikon Eclipse 80i microscope, displayed on a hemocytometer. The coelomocyte types consist of phagocyte (A), crystal (B), morula (C), fusiform (D). A coelomocyte aggregate is shown in (E).

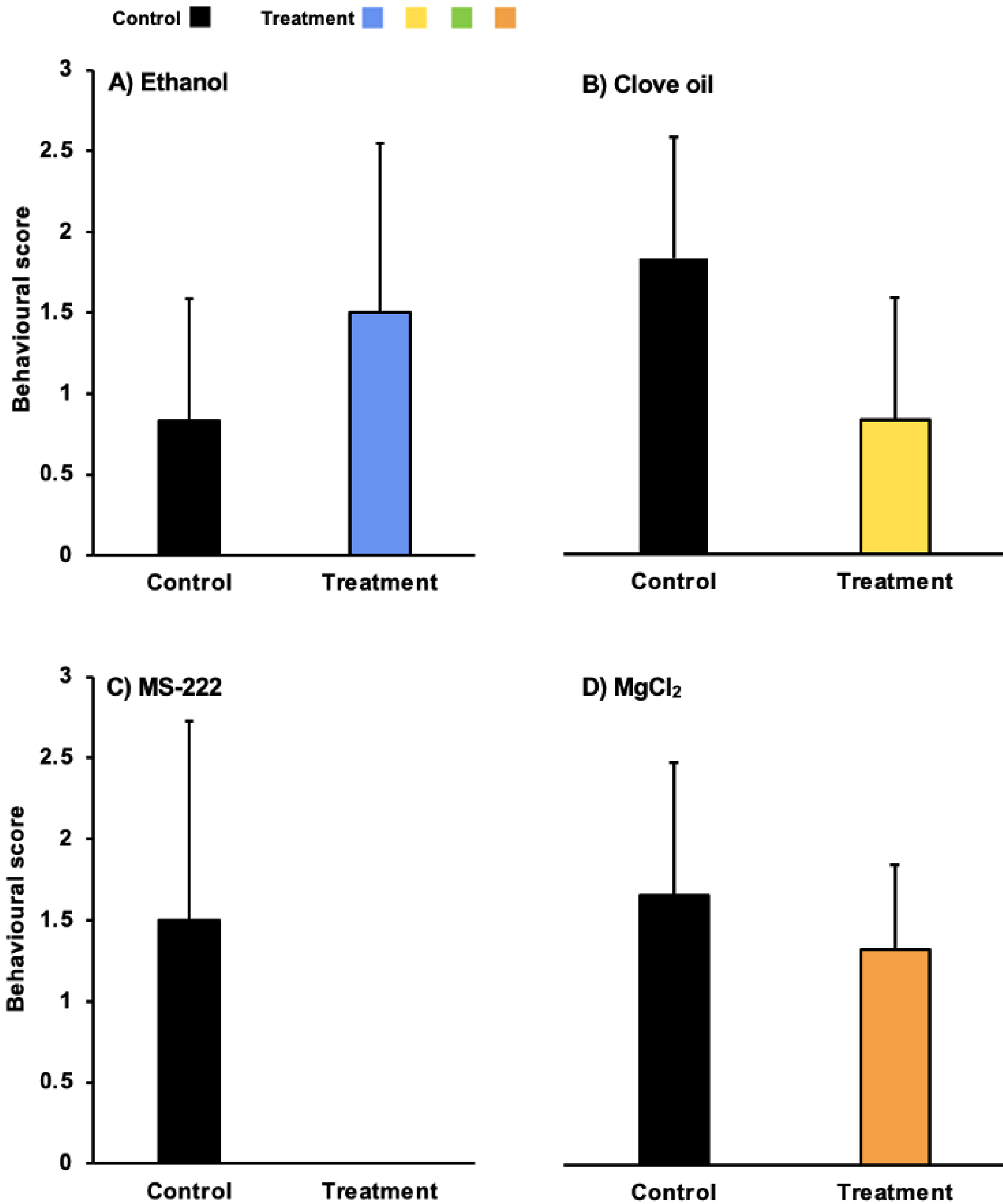

Figure 2. Behavioural scores for control and treatment groups of each chemical, scored as seen in Table 2 (mean±SD, n=6): Ethanol (A), Clove oil (B), MS-222 (C) where the treatment result is zero, and $MgCl_2$ (D). All treatments are shown in different colours to distinguish between the different anesthetics; controls are in black.

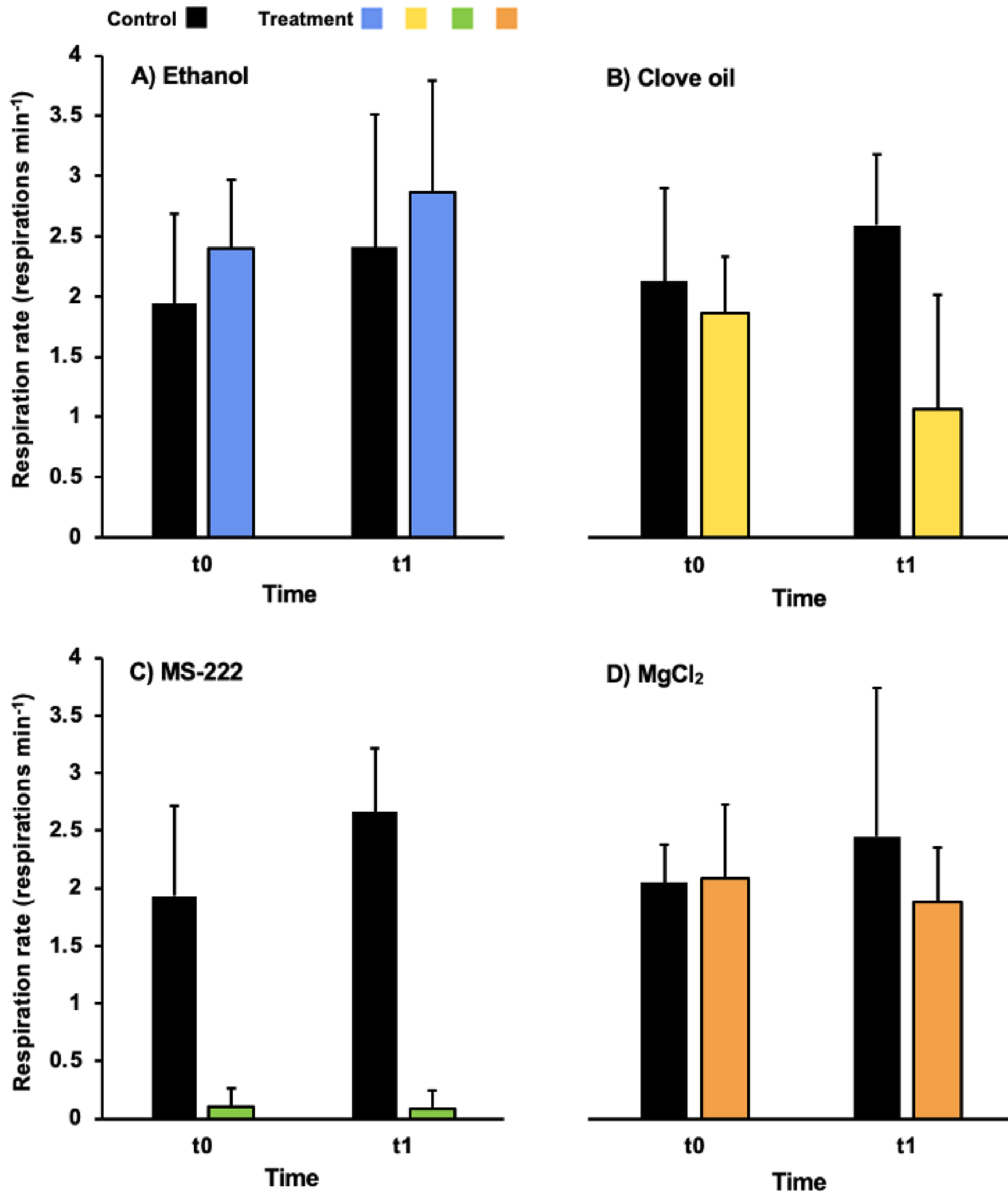

Figure 3. Cloacal respiration rates (mean±SD, n=6; respirations min^{-1}) of control and treatment groups at the beginning (t_0) and end (t_1) of the immersion period in the different chemicals: Ethanol (A), clove oil (B), MS-222 (C), $MgCl_2$ trial (D). Black bars are the control groups and coloured bars are the treatment groups of each anesthetic agent.

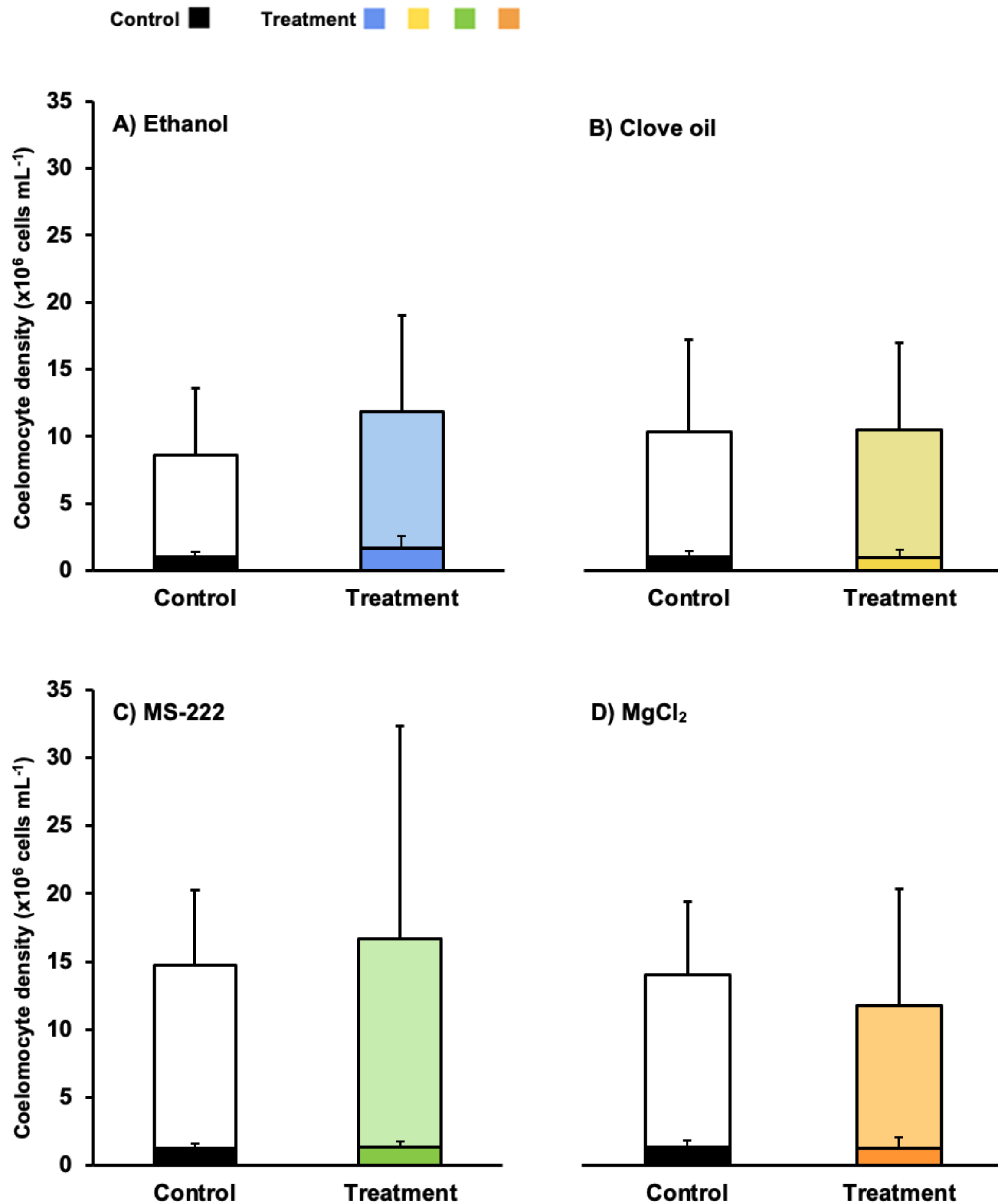

Figure 4. Total coelomocyte densities (cells x 10^6 mL^{-1}) of control and treatment groups for each anesthetic agent (mean±SD, n=6): Ethanol (A), clove oil (B), MS-222 (C) and $MgCl_2$ (D). Each bar is split into two segments, the dark-coloured portion (black for control) representing the free coelomocytes and light-coloured portion (white for control) representing the coelomocytes that are contained within aggregates.

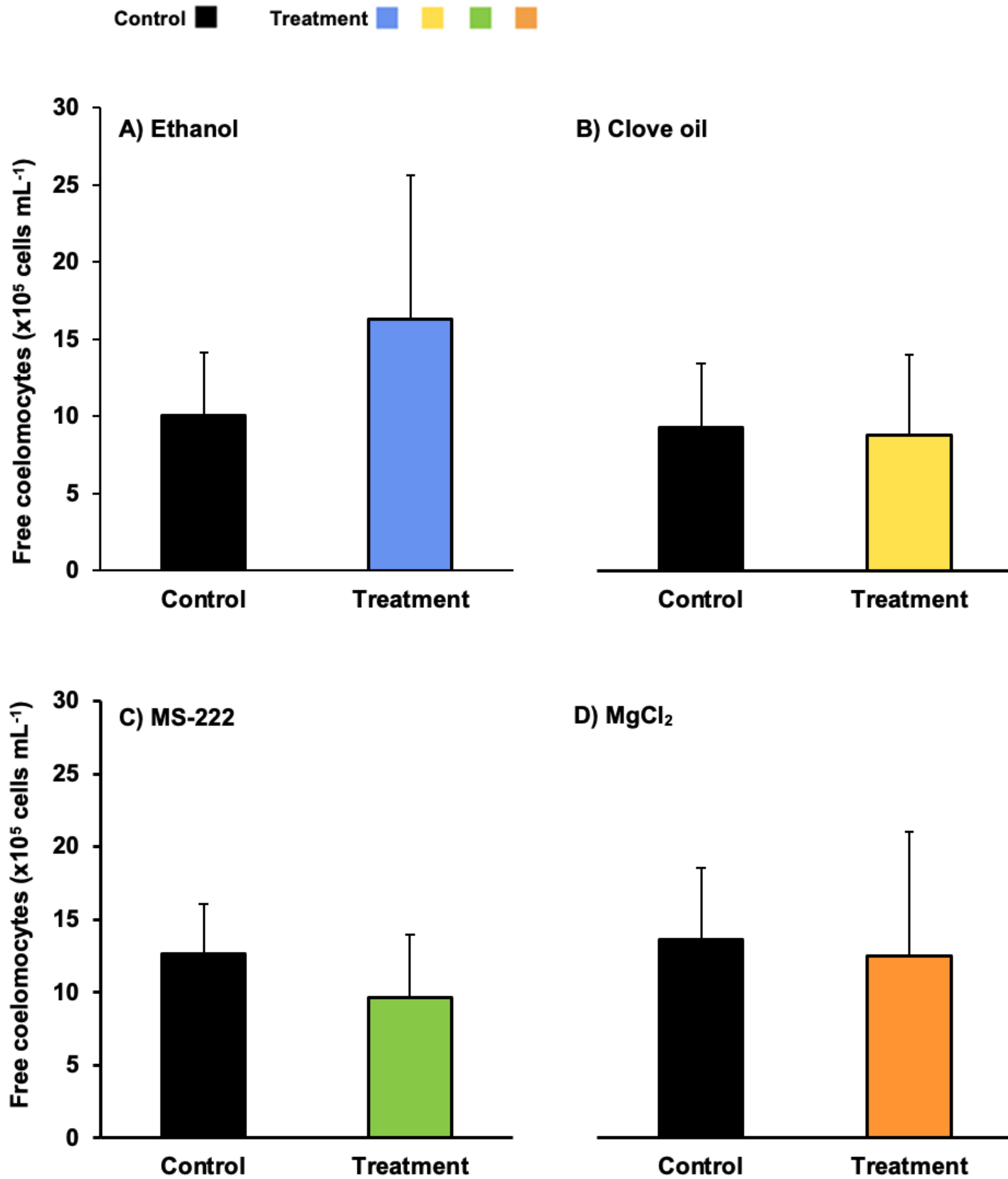

Figure 5. Free coelomocyte densities (cells x 10^5 mL^{-1}) of control and treatment groups for each anesthetic agent (mean±SD, n=6): Ethanol (A), clove oil (B), MS-222 (C) and $MgCl_2$ (D). The treatments are in colour and the controls are in black.

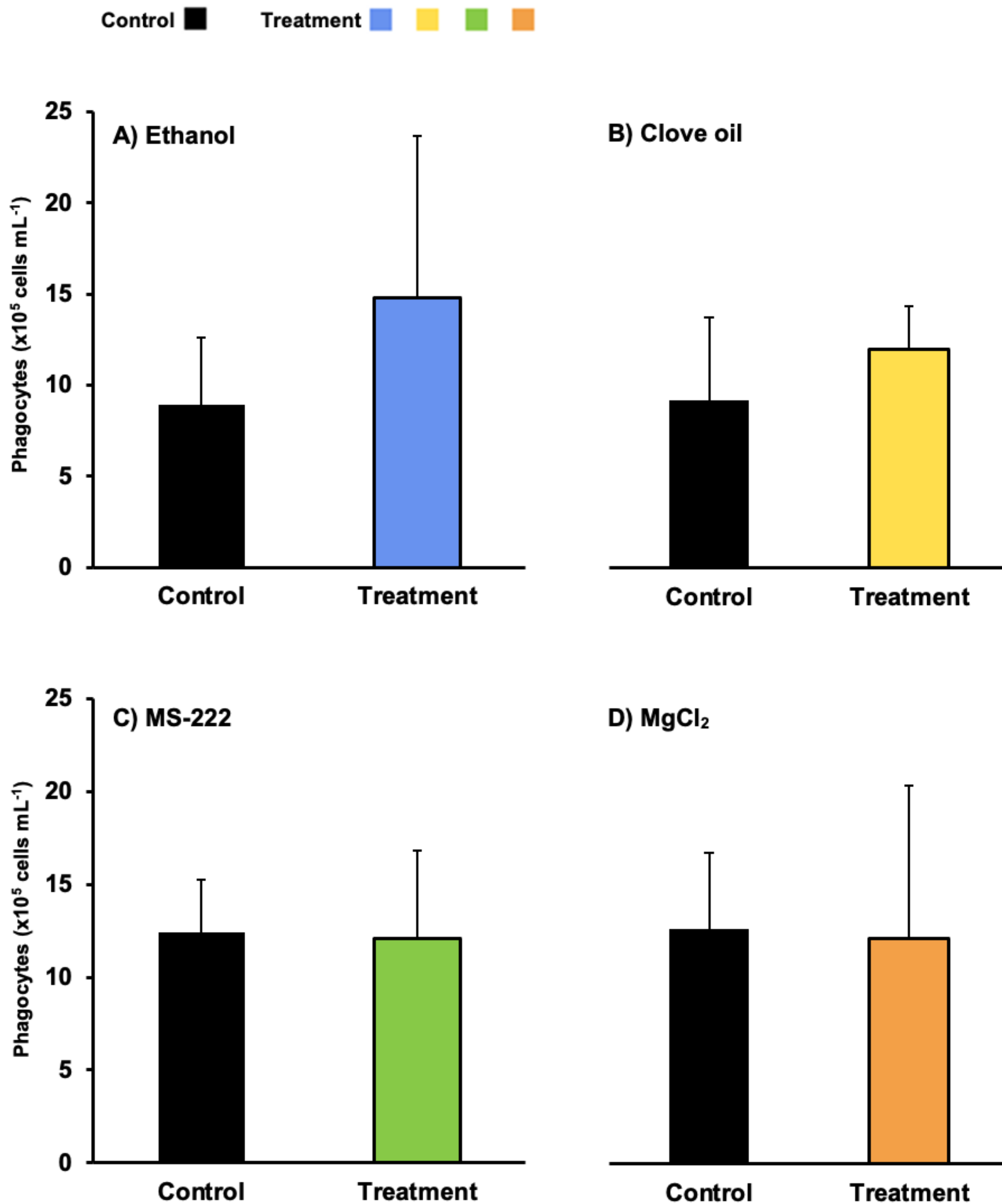

Figure 6. Phagocyte densities (cells x 10^5 mL^{-1}) of control and treatment groups for each chemical (mean±SD, n=6): Ethanol (A), clove oil (B), MS-222 (C) and $MgCl_2$ (D). The treatments are in colour and the controls are in black.

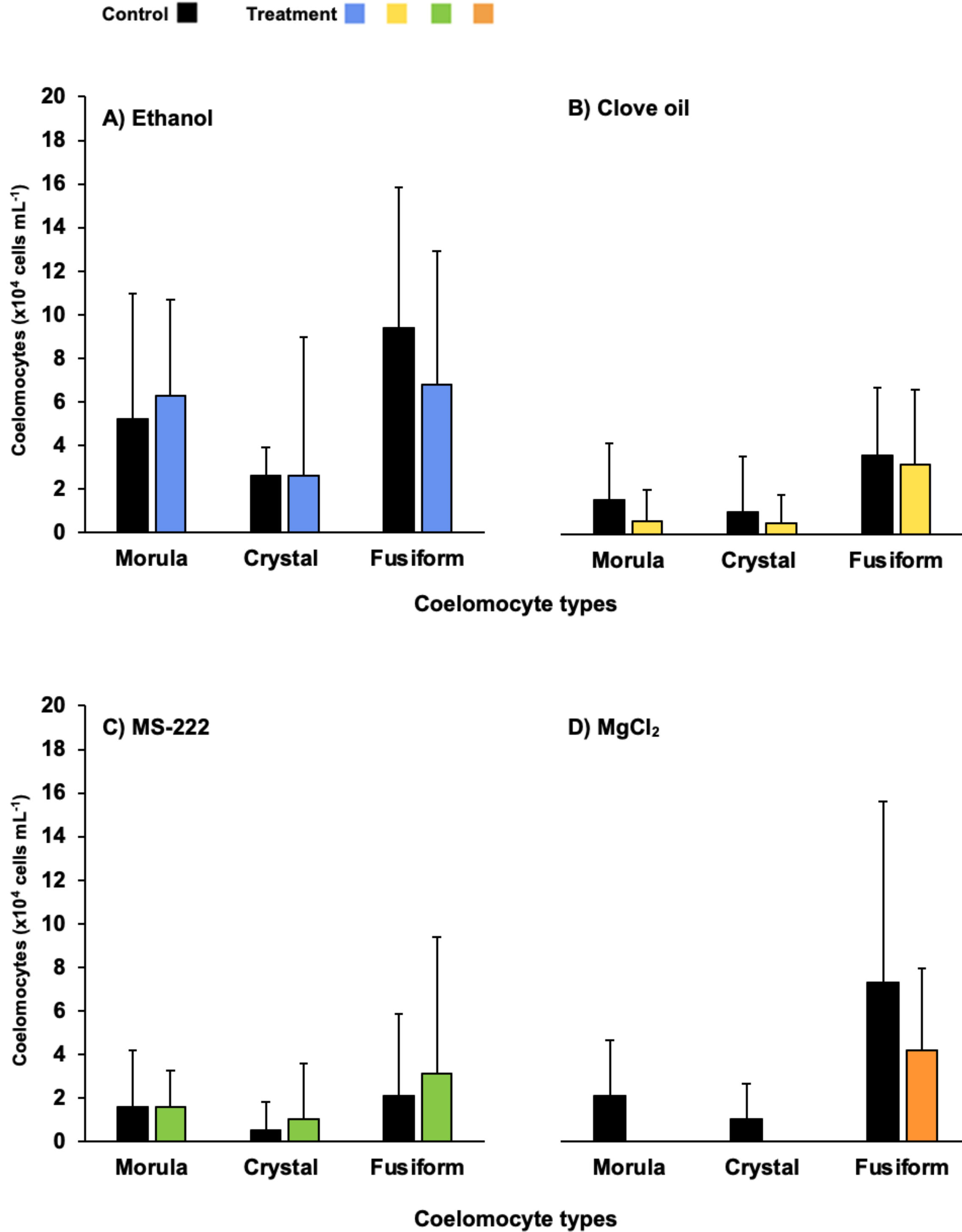

Figure 7. Densities (cells x 10^4 mL^{-1}) of the various free coelomocyte types for each chemical (mean±SD, n=6): Ethanol (A), clove oil (B), MS-222 (C) and $MgCl_2$ (with treatments devoid of morula and crystal cells) (D). The treatments are in colour and the controls are in black.

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