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Research Article

Effect of suspended inorganic matter on fertilization success, embryonic development, larval settlement, and juvenile survival of the vase tunicate *Ciona intestinalis* (Linnaeus, 1767)

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Abstract

Prince Edward Island (PEI), Canada, has seen the introduction and the establishment of many exotic tunicate species since the late 1990's. Exploratory research in the Orwell Bay identified turbidity as a potential factor in this failure of tunicates, and in particular *Ciona intestinalis*, to establish despite multiple unintentional introductions. Laboratory experiments showed significant negative effect of increased suspended inorganic matter on the fertilization success, larval settlement, and the survival of juvenile tunicates. Although the levels of turbidity tested (up to 22 and 32 NTU) reduced all of these processes it did not completely eliminate them. High levels of turbidity have the potential to prevent establishment of non-native tunicates in some locations where establishment would otherwise be expected.

Key words: ascidian; invasive species; laboratory experiment; environmental tolerance; establishment

Introduction

Aquatic invasive species have the capacity to change community structure and function (D'Antonio and Vitousek 1992; Travis 1993; Lambert 2001; Mack et al. 2000; Therriault and Herborg 2008a; Waldner 2008; Shinen and Morgan 2009), and alter essential processes such as sedimentation and nutrient exchange (Locke 2009; Mack et al. 2000; Molnar et al. 2008). Invasive tunicates are an increasing problem (Carver et al. 2003; Lambert and Lambert 1998; Lambert 2001; Lambert 2002; McKindsey et al. 2007), with 57 species listed as invaders of marine and/or estuarine waters worldwide (Locke 2009). These tunicates originate from many locations and are introduced through multiple pathways (Lambert 2001; Molnar et al. 2008; Ramsay et al. 2008). Since the late 1990's, the clubbed tunicate [*Styela clava* (Herdman, 1881)], vase tunicate [*Ciona intestinalis* (Linnaeus, 1767)], violet tunicate [*Botrylloides violaceus* (Oka, 1927)] and golden star tunicate [*Botryllus schlosseri* (Pallas, 1766)], have been introduced into Prince Edward Island (PEI) coastal waters (Thompson and MacNair 2004; MacNair 2005; Locke et al. 2007; Ramsay et al. 2008) where they can dominate the hard-substrate community. In addition to changes to native populations, they can have serious economic impacts for fisheries and the aquaculture industry (Carver et al. 2003; MacNair 2005; Lambert 2007; Locke et al. 2007; Lutz-Collins et al. 2009); e.g., the cultivated mussel industry experienced increased operating costs and equipment fouling (Carver et al. 2003; Leblanc et al. 2003; Thompson and MacNair 2004; Petersen 2007; Daigle and Herbinger 2009). Considerable efforts are being made to mitigate these negative effects.

In 2009, the Canadian mussel aquaculture industry harvested 24,000 tonnes of blue mussels *Mytilus edulis* Linnaeus, 1758, of which 77% was grown in PEI (DFO 2010). This high production requires many artificial structures such as mussel socks, buoys, anchors, and marina structures, (Thompson and MacNair 2004; Locke et al. 2007; Lutz-Collins et al. 2009; Ramsay et al. 2009); all of

which provide the hard substrate required for tunicate establishment (Lambert 2001, 2007; Carver et al. 2006; Tyrrell and Byers 2007). While most PEI coastal waters appear to have optimal conditions for the establishment of introduced tunicate species (Locke et al. 2007; Ramsay et al. 2008; Arsenault et al. 2009), not all aquatic systems have proven to be suitable (Bellas et al. 2003; Dunstan and Johnson 2004; Bourque et al. 2007; Tyrrell and Byers 2007; Therriault and Herborg 2008a,b; Locke 2009). Orwell Bay (including the estuary), located on the south-east side of PEI, has been one such exception. While a few specimens of *S. clava, B. violaceus*, and *B. schlosseri* have been observed their numbers remain very low (Thompson and MacNair 2004; R. Bernier, Fisheries and Oceans Canada, Moncton, NB, pers. comm.). Moreover, *C. intestinalis* has not become established despite heavy colonization of the nearby Brudenell and Montague Estuary complex (Ramsay et al. 2008; Lutz-Collins et al. 2009; Ramsay et al. 2009). The two areas are similar in terms of average salinity and water temperature (McLaughlin, unpublished data) but do differ in terms of water renewal times: 2 to 6 days in Orwell Bay compared to 10 to 24 days in the Brudenell-Montague complex (McLaughlin, unpublished data). The different hydrodynamic characteristics may be responsible for the relatively high turbidity of Orwell Bay (mean ± SE turbidity 15.0 ±52.6 Nephelometric Turbidity Units, NTU) compared to the Brudenell-Montague complex (2.7 ±2.0 NTU). This difference in turbidity may play an important role in the failure of invasive tunicates to establish populations in Orwell Bay.

Elevated turbidity can affect filtration, ingestion rate, and even cause mortality in adult ascidians (Fiala-Médioni 1979; Robbins 1983; Robbins 1984; Robbins 1985a, b; Petersen and Riisgård 1992; Sisgsgaard et al. 2003). There is, however, little information available on the effects of turbidity level on early life stages and development of tunicates. Thus the main goals of this study were to evaluate, in the laboratory, the effect of different levels of suspended inorganic matter on fertilization success, embryonic development, larval settlement, and juvenile survival of *C. intestinalis*. *C. intestinalis* was chosen for our trials because it currently is one of the most problematic species for the aquaculture industry in PEI (Ramsay et al. 2008) and the ease of obtaining viable gametes and producing competent larvae (Cirino et al. 2002; Bellas et al. 2003).

Materials and methods

The inorganic matter used in this study was prepared using commercially available, artificial, soil used to grow aquatic plants (ceramic granules made from 100% Natural Fuller's Earth or Arcillite). The granular Fuller's earth was crushed in a blender, ground further using a mortar and pestle, and then placed into a muffle furnace at 500 °C for 6 hours to remove all organic matter. A stock solution was prepared by mixing the inorganic particles with synthetic seawater at 28 ppt (Instant Ocean, United Pet Group, Virginia, USA). The solution was left undisturbed for 30 minutes and passed through a 20-µm sieve to remove all larger particles. The stock solution was then used to create the desired turbidity levels by dilution. The majority of the particles measured with a Coulter counter (Z2 Coulter counter, Beckman Coulter Canada LP, Ontario, Canada) were between 5 and 10 µm in diameter. Turbidity levels were measured in NTU (Nephelometric Turbidity Unit) using a turbidimeter (Oakton T-100, Eutech Instruments, Singapore). For our, trials 1 NTU = 0.0022 mg/L.

1. Effect of inorganic matter on fertilization success, embryonic development and larval settlement

In October 2010, an experimental setup was designed to test the effect of inorganic matter on the fertilization, early development, and larval settlement of *C. intestinalis*. The experimental units consisted of a 2-L PETE-1 (polyethylene terephthalate) bottle (30 cm height) cut horizontally at 11.5 cm from the bottom (Figure 1). An air stone was inserted into the cap of each bottle and the airflow maintained at 0.757 L/min. Each bottle contained 650 mL of one of three levels of turbidity: 0 NTU (control), 12 NTU and 22 NTU. The turbidity level of 12 NTU was chosen to represent turbidity levels measured in Orwell Bay (Bourque, unpublished data) while 22 NTU was chosen arbitrarily to represent a higher turbidity level. Water temperature was maintained at 17.5 ºC. For each turbidity level, there were initially 15 replicates, which allowed for temporal destructive sampling (5 times) in triplicate.

The bottles were arranged on a sheet of plywood (approximately 100 × 125 cm) in a square formation of 8 by 6 bottles (total of 48 bottles). 45 bottles were completely randomized in 3 blocks (15 bottles per block) in order to control variations associated with bottle location.

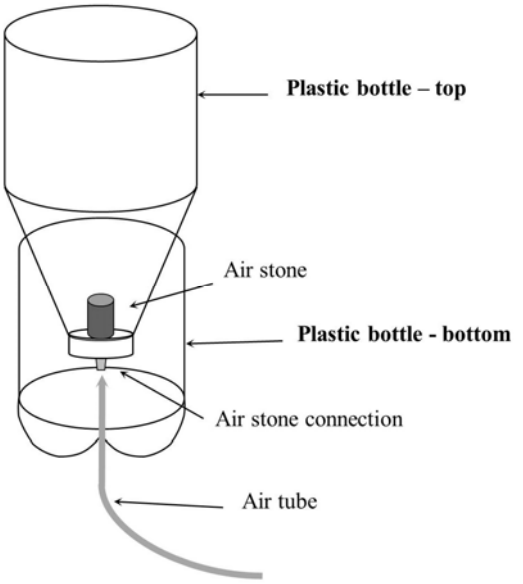

Figure 1. Experimental unit used to test the effect of suspended inorganic matter on the fertilization rate, embryonic development and larval settlement of *Ciona intestinalis*. Each unit consisted of a 2 litre plastic bottle cut in halve with an air stone inserted into the cap to create water movement inside the bottle.

Three additional bottles (one at each treatment level) were used to monitor temperature, salinity and turbidity levels without disturbing the experimental units. Turbidity levels, salinity and temperature were measured frequently using a turbidimeter (Oakton T-100, OAKTON Instruments, Illinois, USA), an Opticon Series salinity refractometer (model FG100sa, Sybon Scientific Instruments, Maryland, USA) and a thermometer (Enviro-safe, Thermo Fisher Scientific, Massachusetts, USA) respectively. A natural photoperiod corresponding to field conditions was set in the room.

Mature *C. intestinalis* were collected on a mussel aquaculture site in Brudenell River, PEI, and maintained in laboratory aquaria until dissection. We used the protocol describe in Cirino et al. (2002) to obtain viable gametes. Briefly, gametes were collected from multiple specimens and pooled together. An equal volume of egg and salt water solution was introduced into each bottle followed by 15 drops of diluted sperm. There were no water changes made after sperm introduction to avoid disturbance of post-fertilization development processes.

Samples were collected from three replicates of each treatment at five intervals post introduction of sperm. Sampling times were: 18 hours, 30 hours, 42 hours, 54 hours, and 66 hours. The contents of each bottle were emptied onto a 63 µm sieve and the material retained was placed into a 20 ml bottle with 4% formalin solution for later analysis. The bottle was then cut in half and examined under a stereomicroscope to detect settlement on its surface. For all samples, the developmental stages were identified and counted.

Developmental stages were identified based on published images and descriptions (Na and Lee 1977; Cirino et al. 2002; Chiba et al. 2004). Five groups were defined: 1) abnormal development (including abnormal presentation of all development stages); 2) unfertilized eggs; 3) developing stage (2-cells, 4 cells, 8 cells, 16 cells, 32 cells, gastrula, tail-bud embryo, pre-hatching embryo, and free swimming tadpole larva); 4) fixed metamorphosis stage (settlement on the bottle including fixed tadpole larva, tail resorption larva, metamorphosed larva, and individuals having two lateral siphons); and 5) not fixed metamorphosis stage (similar to group 4 but unattached).

2. Effect of inorganic matter on juvenile survival

In October 2010, a different experimental setup was used to test the effect of inorganic matter on the survival of juvenile tunicates (i.e., not sexually mature). The trials were conducted in nine 118-L polyethylene tanks (Figure 2b). Each tank had a cylindrical section with a 48.3 cm internal diameter (ID) and height of 53.3 cm from the top to the start in the funnel. The funnel section had a height of 20.3 cm. There were three units each consisting of three tanks (Figure 2a).

To maintain turbidity levels, water was pumped from the bottom of the tank to the top through a 1.27 cm ID vinyl tubing and then flowed through a 1.27 cm ID rigid chlorinated polyvinyl chloride (CPVC) pipe that extended 21.6 cm into the tank. An elbow inserted at the end of the rigid pipe created a circular current.

Each tank was filled with 55 liters of synthetic seawater (28 ppt) at one of three turbidity levels: 0, 22 and 32 NTU. Three turbidity levels were assigned in triplicate to the 9 experimental tanks following a Latin square to control for variation associated with tank location in the support units. Turbidity levels chosen were slightly higher than the previous trial because turbidity levels dropped approximately 75% in treatments over a 24 hour period. Turbidity was monitored using an AquafluorTM Handheld Fluorometer/Turbidimeter (Turner Design inc., California, USA) with both turbidity and *in vivo* chlorophyll *a* channels.

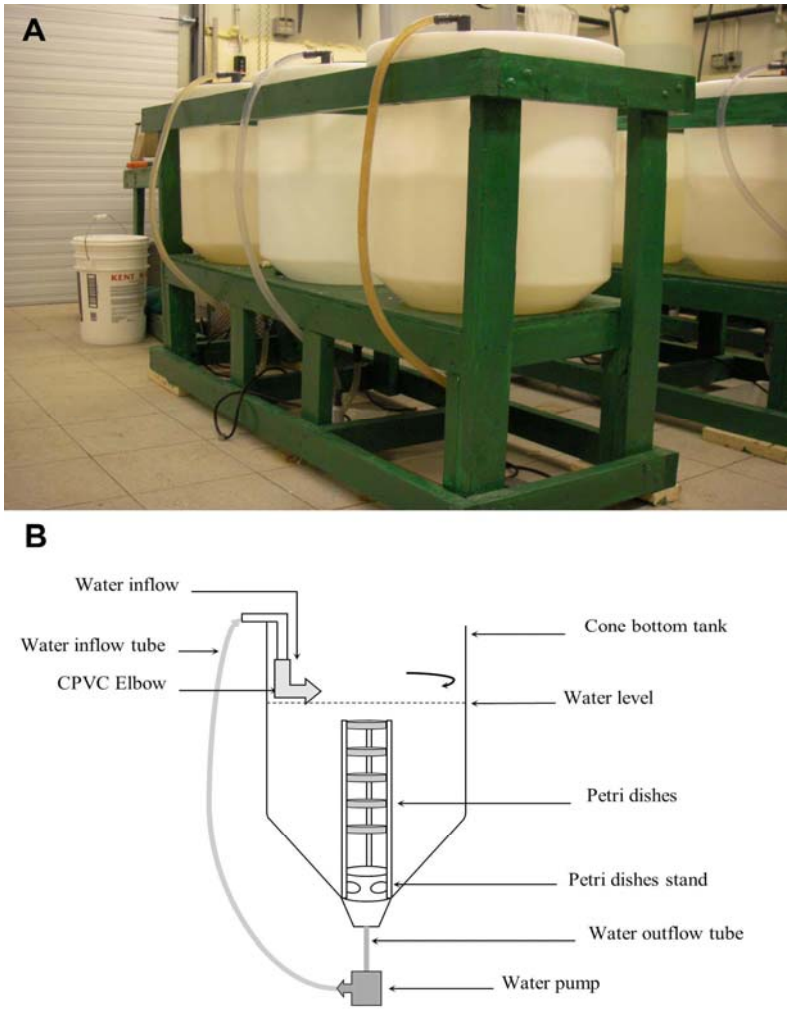

Figure 2. A) Wooden stand constructed to maintain tanks vertically. Each of the 3 stands supported 3 experimental units. B) Experimental unit used to test the effect of suspended inorganic matter on juvenile *Ciona intestinalis* survival. Each unit consisted of a 118 litre cone bottom tank attached to a circulation pump to create water flow around the Petri dishes attached in the center.

Table 1. Mean initial count, final count and percentage of the loss of eggs and other development stages of *Ciona intestinalis* for each turbidity treatment and each block.

Turbidity treatment	blocs	n	Initial count	Final count	% loss (SE)
0 NTU	bloc 1	5	283.4	140.8	49.3 (10.5)
	bloc 2	5	266.0	140.6	45.2 (8.1)
	bloc 3	5	287.4	165.4	42.1 (7.7)
Treatment mean			278.9	148.9	45.5 (8.7)
12 NTU	bloc 1	5	276.8	140.8	49.5 (8.2)
	bloc 2	5	258.0	124.2	51.7 (7.3)
	bloc 3	5	290.2	150.0	48.2 (6.9)
Treatment mean			275.0	138.3	49.8 (7.5)
22 NTU	bloc 1	5	279.6	139.2	49.8 (7.6)
	bloc 2	5	275.0	145.0	46.8 (11.2)
	bloc 3	5	275.8	113.4	61.4 (11.6)
Treatment mean			276.8	132.5	52.67 (10.2)
Overall Total			279.9	139.9	49.3

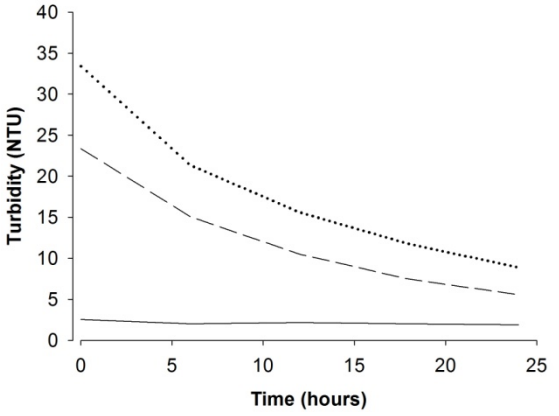

Figure 3. Decrease in turbidity levels in experimental tanks over a 24 hours period. Mean decrease of turbidity level is presented for each treatments (solid line = 0 NTU; long dash line = 22 NTU; dotted line = 32 NTU), n=3.

Turbidity levels were quantified every 6 hours for 24 hours on days 0, 13, and 18 to document this decline (Figure 3). Turbidity was constant for the 0 NTU treatment (as expected) but decreased steadily for the 22 NTU and 32 NTU treatments. Turbidity levels were, however, greater than 12 NTU for approximately 10 hours for the 22 NTU treatment and 18 hours for the 32 NTU treatment. Because of this decrease, the tanks were manually mixed every 24 hours to raise turbidity to the required level. All tanks were mixed similarly, including the 0 NTU tanks. At times, it was necessary to add additional stock solution of suspended Fuller's earth.

Viable *C. intestinalis* larvae were produced as described in the previous section (based on methods of Cirino et al. 2002). Competent larvae were transferred to 45 Petri dishes (85 × 15 mm diameter) and topped with seawater to obtain a total volume of 30 ml. The dishes were then placed in the dark at a temperature of 22ºC (Tursi 1980). Once larvae had attached to the dish, usually within a few minutes or hours after transfer, they were maintained at the same temperature for 5 days with a natural photoperiod cycle until both lateral siphons were fully developed and the first pair of functional stigmata were present (Cirino et al. 2002). At this stage, juveniles require a food source. Every 24 hours, until trials were run, complete water changes were performed and food (a mix of equal parts of *Isochrysis galbana*, *Pavlova lutheri,* and *Nannochloropsis occulata*) was added at an average concentration of 7,000 cell/mL to simulate natural concentrations (Bates and Strain 2006; L. Comeau, Fisheries and Oceans Canada, pers. comm.). Counts of juvenile tunicates in each Petri dish were conducted with a stereomicroscope prior to being added to the experimental units.

Five Petri dishes with juveniles were placed into each tank using supports built with commercially available plastic egg grate (used for light diffusion), which possess square openings. These were cut into small rectangular pieces of 42 openings (i.e. 2 × 21). Three of these supports were attached to a 7.62 cm PVC (polyvinyl chloride) pipe union fitting using plastic cable ties. Slots in the support allowed the Petri dishes to be stacked vertically, face down, with equal spacing between dishes. Large plastic cable ties were used to retain Petri dishes in the supports. Each stack was secured to the bottom of the conical tank for the duration of the trials (Figure 2b).

To meet the food requirements of the juvenile tunicates during the trial, phytoplankton (the same mix of equal parts of *Isochrysis galbana*, *Pavlova lutheri*, and *Nannochloropsis occulata*) was added to each tank and maintained at a concentration of about 7000 cell/mL. Food levels were monitored daily based on chlorophyll *a* levels measured with the Aquafluor Handheld Fluorometer/Turbidimeter and calibrated for each turbidity level.

Water temperature varied between 17.0 ºC to 19.5 ºC. Ammonia, nitrate, and pH were monitored with standard aquarium test kits (Rolf C. Hagen Inc., Quebec, Canada) but no significant changes were observed. Salinity was monitored regularly with a refractometer and when it increased (due to evaporation), distilled water was slowly added to the tank.

One Petri dish was removed randomly from each experimental unit on each of days 3, 4, 6, 11, and 17. The first sampling was chosen on day 3 to allow time for treatments to affect the feeding behaviour of the juvenile tunicates while sampling on days 4 and 6 was done because high mortality had been expected. Because initial mortality was low, sampling was then delayed to days 11 and 17. At each sampling time, a Petri dish was removed from each tank and examined under a stereomicroscope to assess the survival of juvenile tunicates. Each Petri dish that was sampled (destructive model) was replaced with a clean dish to avoid changes in water circulation within the experimental units.

Statistical analysis

Prior to evaluating the response of fertilization success, embryonic development and larval settlement over time to the three turbidity treatments, we noticed a loss of eggs and other development stages in the bottles when compared to our initial egg counts (Table 1). For this reason, a fixed effect one-way ANOVA with completely randomized block was conducted to verify if there was a significant difference between treatments, in terms of percentage of tunicate losses (eggs and other stages). Those results showed no significant difference between treatments; therefore, we proceeded to further analysis.

The outcome of the experiment (Y = the number of observation in development stage k) is a multinomial variable with k categories (k = 5 groups of development stage). In addition, the categories were ordered and this type of data is best analyzed with ordinal regression (Ananth and Kleinbaum 1997; Scott et al. 1997; Armstrong and Sloan 1989; Guisan and Harrell 2000; Cole and Ananth 2001). We chose the continuation ratio model (CR) (Abreu et al. 2008; Abreu et al. 2009) because the animals observed have to pass through one category to reach the next one and cannot revert (Cole and Ananth 2001). The CR model is based on the conditional probabilities of being in category j given that one is in category j or higher. So if Y is a categorical response with k ordered categories, then the conditional probabilities (2) are:

$$\pi_j = \Pr(Y = j \mid Y \geq j, X=x) \quad (2)$$

with $j = 1, 2,\ldots, k$, X is a vector of explanatory variables,

$$Pr(Y \geq j, X = x) = \sum_{j}^{k} \Pr(Y = j, X = x)$$

and $Pr(Y = k|Y \geq k, X = x) = 1$ for the last category. The log-odds or logit of the probabilities is linearly related to the predictor variables by:

$$logit\ \pi_j = \log\left[\frac{\Pr(Y = y_j|x}{\Pr(Y \geq y_j|x}\right] = \alpha_j - x'\beta_j \quad (3)$$

where for each category (3) ($j = 1, \ldots, k$), α_j is the intercept and β_j is the coefficient for the X explanatory variables. The odds of an event happening (4) are given by:

$$O_i = \frac{\Pr(Y=y_i|x)}{\Pr(Y\geq y_i|x)} = exp\{\alpha_j - x^i\beta_j\} \quad (4)$$

To compare different levels of a factor X_l (ex: level A vs. level B), the odds ratio is used due to its ease of interpretation. It is calculated with the following formula:

$$OR_j = \frac{\frac{\Pr\left(Y=y_i|x_l^{(A)}\right)}{\Pr\left(Y\geq y_i|x_l^{(A)}\right)}}{\frac{\Pr\left(Y=y_i|x_l^{(B)}\right)}{\Pr\left(Y\geq y_i|x_l^{(B)}\right)}} = \frac{odds^{(A)}}{odds^{(B)}} = \exp\{-\beta_{jl}(x_l^{(A)} - x_l^{(B)})\} \quad (5)$$

The odds ratio (5) quantifies the odds of an observation in factor level A being in category j condition on being in category j or higher, compared to the same odds in factor level B.

The abnormal category includes abnormalities from all stages of development. To move on to the next stage of development, one has to be normal. We therefore chose to use the abnormal category as the first one. Using it as the last category would imply that the eggs went through all stages of development and were abnormal in the end, which was not the case in the experiment. The order of the other categories followed the natural process: unfertilized eggs, embryonic development, fixed metamorphosis and not fixed metamorphosis.

The extended CR model, which allows for different slopes for each category of the response, was more appropriate than a model with parallel slopes. A saturated model was fitted with time (continuous), treatment (categorical with 3 levels: 0, 12 and 22 NTU), block and an interaction between time and treatment as the covariables. Because we do not know the relationship between the gametes, turbidity and time before the first sampling time, the intercept was adjusted to be the time of the first sampling (18 hours), so that it would be meaningful in the interpretation of the results. The significance of factors was determined by comparing the deviances of the saturated model and a model that did not include the factor with a likelihood ratio test (Armstrong and Sloan 1989). The models were fit in R (R Development Core Team 2012) using the vglm() function of the VGAM (Yee 2012) package. Treatments were compared using the estimate (ß) and the OR (odds ratios). The output of the analysis does not give any P value. The z-value (t) and the CI (confidence interval, 95%) were used to determine the threshold of significance for this experiment. When the z-value is between 2 and -2, and when the CI includes 0, it is not statistically significant.

In the second experiment, the survival rate over time of juvenile tunicates exposed to three

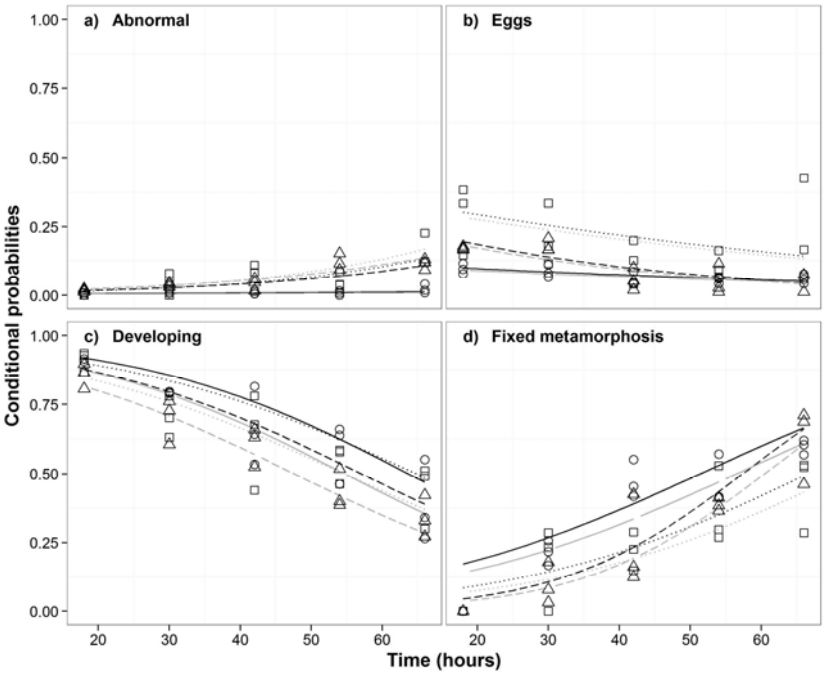

Figure 4. Conditional probabilities of the 4 groups of development stages of *Ciona intestinalis* from the continuation ratio model (solid line and circles = 0 NTU; dotted line and triangles = 12 NTU; long dash line and squares = 22 NTU), n=3. The two different blocks included in the analysis are the lines in black and grey.

turbidity levels was evaluated. A mixed logistic regression with binomial errors and link logit (Quinn and Keough 2002) was used with turbidity as a categorical factor and time as a continuous factor. To address overdispersion in the data, we used the quasibinomial family in the nlme (Pinheiro et al. 2013) package (glm function) from the R software. Treatments were compared using the function glht from the multcomp (Hothorn et al. 2008) package. $P < 0.05$ was used to determined threshold of significance.

Results

1. Effect of inorganic matter on fertilization success embryonic development and larval settlement

The loss of eggs and other development stages (Table 1) was the same for all turbidity treatments in all blocks (block*treatment: $F_{2.38} = 0.49$, $P = 0.6155$). There was also no significant difference between treatments during the experiment ($F_{1.38} = 0.12$, $P = 0.7312$). There was a mean loss of 49 % of eggs and early development stages of tunicates throughout the trial. We assume that the loss of tunicates has been distributed and randomized to every stage of development during the experiment.

For the continuous ratio logistic model, a continuation model with different slopes for each category of the outcome was a better fit than a model with equal slopes ($\chi^2 = 1269$, df = 21, $P < 0.001$). Two of the three blocks were not statistically different therefore they were combined in the final model. The block factor (with 2 levels) was statistically significant ($\chi^2 = 56.0$, df = 4, $P < 0.001$). The interaction between time and treatment was also significant ($\chi^2 = 26.2$, df = 8, P = 0.001) (Figure 4, Table 2). Several of the results that were considered statistically significant were very close to the threshold of significance ($|z| > 2$ and 95 % CI not containing 0).

Eighteen hours after female gametes were in contact with the male gametes, the probability of abnormal development increased with an increase of turbidity levels. Control bottles (0 NTU) showed very low abnormality levels, which were constant over sampling times. The odds of tunicate gametes or a more advanced development stage being abnormal in the 12 NTU treatment were 3.0 times higher than in the 0 NTU treatment, and 2.6 times higher in the 22 NTU treatment compared to the 0 NTU. There was no statistically significant difference observed between the 12 and 22 NTU treatments. The odds of being abnormal did not change with time (slope = 0) for the 0 NTU and

Table 2. Estimated coefficients, standard errors, z-values, odds ratios and 95% confidence intervals of the continuation ratio model for juvenile *Ciona intestinalis* using time, treatment, block and time X treatment as co-variables. Here, the estimate value of treatment 0 NTU is equal to the estimate value of the intercept. Significant factor are in bold.

	$\hat{\beta}$(SE)	z-value	95% CI	$\widehat{OR}$	95% CI
a) Abnormal					
Intercept	**-5.120 (0.398)**	**-12.87**	**-5.899, -4.340**		
Time	0.014 (0.014)	1.05	-0.012, 0.041	1.014	0.988, 1.041
12NTU	**1.099 (0.431)**	**2.55**	**0.255, 1.944**	**3.002**	**1.290, 6.987**
22 NTU	**0.960 (0.436)**	**2.20**	**0.105, 1.815**	**2.611**	**1.111, 6.139**
Block (2 levels)	0.266 (0.147)	1.82	-0.021, 0.554	1.305	0.979, 1.740
Time*12 NTU	0.026 (0.015)	1.76	-0.003, 0.055	1.026	0.997, 1.056
Time*22 NTU	**0.034 (0.015)**	**2.28**	**0.005, 0.063**	**1.034**	**1.005, 1.065**
12 NTU vs 22NTU	-0.140 (0.290)	-0.48	-0.708, 0.429	0.870	0.493, 1.536
Time*12NTU vs Time*22NTU	0.008 (0.008)	0.94	-0.009, 0.024	1.008	0.992, 1.025
b) Eggs if normal					
Intercept	**-2.199 (0.136)**	**-16.20**	**-2.465, -1.933**		
Time	**-0.014 (0.005)**	**-2.68**	**-0.025, -0.004**	**0.986**	**0.975, 0.996**
12 NTU	**0.788 (0.155)**	**5.10**	**0.485, 1.091**	**2.198**	**1.624, 2.976**
22 NTU	**1.364 (0.146)**	**9.36**	**1.078, 1.650**	**3.911**	**2.940, 5.205**
Block (2 levels)	-0.089 (0.081)	-1.10	-0.247, 0.069	0.915	0.781, 1.072
Time*12 NTU	**-0.019 (0.007)**	**-2.62**	**-0.034, -0.005**	**0.981**	**0.967, 0.995**
Time*22 NTU	-0.005 (0.006)	-0.82	-0.018, 0.007	0.995	0.982, 1.007
12 NTU vs 22NTU	**0.576 (0.121)**	**4.76**	**0.339, 0.814**	**1.780**	**1.403, 2.257**
Time*12NTU vs Time*22NTU	**0.014 (0.006)**	**2.29**	**0.002, 0.026**	**1.014**	**1.002, 1.026**
c) Developing if fertilized					
Intercept	**2.414 (0.114)**	**21.14**	**2.190, 2.637**		
Time	**-0.053 (0.003)**	**-15.27**	**-0.060, -0.046**	**0.949**	**0.942, 0.955**
12 NTU	**-0.455 (0.136)**	**-3.33**	**-0.722, -0.187**	**0.634**	**0.486, 0.829**
22 NTU	-0.226 (0.147)	-1.53	-0.515, 0.063	0.798	0.598, 1.065
Block (2 levels)	**-0.474 (0.070)**	**-6.73**	**-0.612, -0.336**	**0.623**	**0.542, 0.715**
Time*12 NTU	-0.003 (0.005)	0.56	-0.007, -0.012	1.003	0.993, 1.012
Time*22 NTU	0.006 (0.005)	1.21	-0.004, 0.016	1.006	0.996, 1.016
12 NTU vs 22NTU	0.229 (0.143)	1.60	-0.051, 0.509	1.257	0.950, 1.663
Time*12NTU vs Time*22NTU	0.003 (0.005)	0.69	-0.006, 0.013	1.003	0.994, 1.013
d) Fixed if developed					
Intercept	**-1.570 (0.233)**	**-6.74**	**-2.027, -1.114**		
Time	**0.047 (0.006)**	**7.48**	**0.035, 0.059**	**1.048**	**1.035, 1.061**
12 NTU	**-1.479 (0.352)**	**-4.20**	**-2.169, -0.790**	**0.228**	**0.114, 0.454**
22 NTU	**-0.799 (0.366)**	**-2.18**	**-1.516, -0.082**	**0.450**	**0.219, 0.922**
Block (2 levels)	-0.240 (0.126)	-1.91	-0.486, 0.007	0.787	0.615, 1.007
Time*12 NTU	**0.030 (0.010)**	**3.12**	**0.011, 0.050**	**1.031**	**1.011, 1.051**
Time*22 NTU	0.002 (0.010)	0.17	-0.018, 0.022	1.022	0.982, 1.022
12 NTU vs 22NTU	0.680 (0.409)	1.66	-0.122, 1.483	1.975	0.885, 4.406
Time*12NTU vs Time*22NTU	**-0.029 (0.011)**	**-2.62**	**-0.050, -0.007**	**0.972**	**0.951, 0.993**

Table 3. Results of the mixed logistic regression with binomial errors and link logit with turbidity as a categorical factor and time as a continuous factor to evaluate the effect of the treatments on the survival of juvenile *Ciona intestinalis*. The estimate value of 0 NTU is equal to the estimate value of the intercept. Significant factor are in bold.

Effect	Estimate, ß (SE)	DF	t Value	Pr > ItI
Intercept	**2.305 (0.336)**	**41**	**6.857**	**<0.0001**
Time	**-0.155 (0.028)**	**41**	**-5.55**	**<0.0001**
22 NTU	**-2.024 (0.319)**	**41**	**-6.344**	**<0.0001**
32 NTU	**-1.687(0.307)**	**41**	**-5.496**	**<0.0001**
22 NTU vs. 32 NTU	0.337 (0.293)	41	1.149	0.484

12 NTU treatments. However, for every additional hour after 18 hours, the odds of being abnormal increased by 4.9% at 22 NTU (Figure 4a, Table 2a).

Turbidity appeared to reduce fertilization success; however, fertilization normally occurs prior to 18 hours. At 18 hours, the odds of observing unfertilized eggs at 12 and 22 NTU were respectively 2.2 and 3.9 times higher than in the 0 NTU treatment. The differences between treatments were statistically significant (Table 2b). After 18 hours, if an egg had not been fertilized, the chances of fertilization were low. At 0 and 22 NTU the odds of observing unfertilized eggs decreased by 1.4 % per hour while at 12 NTU they decreased by 3.3 %. The fertilization success in the 12 NTU treatment, however, increased over time to eventually reach the same values as the 0 NTU treatment, sometime after 50 hours. Although the 22 NTU treatment fertilization success increased over the study period, it was consistently lower than in the 0 and 12 NTU treatments (Figure 4b, Table 2b).

Fertilized tunicate eggs (developing stages) metamorphosed into juveniles at the same rate in all turbidity levels. The likelihood of observing eggs in any development stage decreased by 5.1% per hour in all treatments (Figure 4c, Table 2c). At 18 hours, egg development stage observations were 0.63 times less likely at 12 NTU than at 0 NTU. However, the odds of observing development stages were similar between 0 and 22 NTU and as well as between 12 and 22 NTU. Additionally, there was a significant difference between blocks.

The odds that a larva will settle on a hard surface and metamorphose into a juvenile were 4.4 and 2.2 times higher at 0 NTU than at 12 NTU and 22 NTU, respectively (Figure 4d, Table 2d). There was no significant difference between 12 and 22 NTU treatments. The likelihood that a larva will settle and metamorphose increased by 4.8% for every additional hour after 18 hours at 0 NTU and 22 NTU. The same odds increased by 8.0% in the 12 NTU treatment. The odds at 22 NTU was significantly different than the odds at 12 NTU for every additional hour after time 18 h, increasing by 2.8 %.

2. Effect of inorganic matter on juvenile survival

Turbidity levels and exposure time influenced juvenile tunicate survival. There were significant differences between turbidity treatments ($F_{2,41}$ = 25.58, $P < 0.0001$). High mortality was observed between days 0 and 3 for treatments of 22 and 32

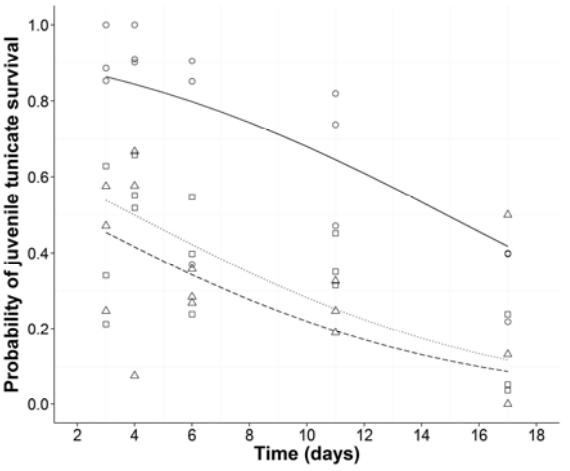

Figure 5. Probability of juvenile *Ciona intestinalis* survival when exposed to different levels of inorganic matter (solid line and circles = 0 NTU; long dash line and triangles = 22 NTU; dotted line and squares = 32 NTU), n = 3.

NTU. The odds of surviving in 0 NTU were 7.6 and 5.4 times higher than the odds in 22 NTU and 32 NTU, respectively, while the 22 NTU and 32 NTU treatments did not differ (Figure 5 and Table 3). The mortality rate differed from zero (14% per day; $F_{1,41}$ = 34.93, $P < 0.0001$) but did not differ between turbidity levels based on the lack of a significant interaction between time and treatment ($F_{2,41}$ = 0.96, P = 0.3917).

Discussion

High suspended inorganic matter concentrations appear to reduce the chances that gametes will develop into adult *C. intestinalis*. However, not all stages of development were affected the same way. Abnormalities did not seem to be an issue for fertilization. Even though our results show that turbidity increases abnormal development in terms of physical appearance (e.g. shape, texture and color), the probability of being abnormal remained extremely low at 18 hours. The slight increase with time suggests that the effects of turbidity are more likely to occur at later stages of development. Because the abnormalities were not associated to specific developmental stages, we cannot confirm this hypothesis. The lack of water change to remove excess sperm could have caused polyspermy, which can affect normal development of *C. intestinalis* (Cirino et al. 2002; Bellas et al. 2003). However, no significant abnormal development was observed in the 0 NTU

treatment; therefore, it is highly unlikely that polyspermy contributed to the occurrence of abnormalities. Furthermore, our results show that increased turbidity levels interfered with the fertilization process, which should have reduced the incidence of polyspermy at the highest turbidity level.

Based on counts of unfertilized eggs, high turbidity seemed to impede the fertilization process. However, our study could not demonstrate how or when turbidity affects fertilization because most of the fertilization occurred before our first sampling (18 hours after gametes contact). It should, however, be noted that our trials were conducted in small closed systems, which increases chance of contact between gametes. It is therefore likely that in a natural environment the dilution of gametes plays an additional role on fertilization success. The likelihood of fertilization occurring decreases considerably with increased gamete dilution with most broadcast spawning marine invertebrate (Oliver and Babcock 1992; Havenhand and Styan 2010); hence, most fertilization occurs shortly after gamete release. It is likely that in a natural environment we would see permanent decreases of fertilization more closely resembling results at the 18 hour sampling time. Consequently, it is improbable that fertilization at 12 NTU would attain the same levels as in the control treatment. Several factors may play a role in the reduction of fertilization in *C. intestinalis* in the presence of high turbidity levels. Some authors suggest an alteration of the sperm motility in the water column (Yoshida et al. 1993; Havenhand and Styan 2010). Others link this reduced fertilisation to egg recognition difficulties by sperm (Lambert 1982; Miller 1982; Svane and Young 1989; Fukumoto 1990a, b; Yoshida et al. 1993). Lastly, gametes can be physical altered to prevent the fusion of gametes (Fukumoto 1990a, b).

Although a significant decrease of the probability of observing some development stages was revealed at 12 NTU, higher turbidity levels did not show any significant impact on embryonic development. This coupled with the absence of significant difference between the 12 and 22 NTU treatments lead to the conclusion that turbidity had a minimal effect on embryonic development in our study. Furthermore, the probability of observing embryonic and larvae development decreased similarly in all treatments over time. Thus the decrease can be attributed to metamorphosis into juveniles and increased turbidity did not measurably affect the development success.

Generally, swimming tadpole larvae attach to a hard substrate within hours or days after hatching (Cirino et al. 2002; Cloney 1982; McHenry 2005) and metamorphose into juveniles. Because we observed a steady increase of settlement of *C. intestinalis* over time for all treatments, we think larvae gradually settled as opportunity became available. Even though the larvae were provided with oblique and vertical surfaces and a natural photoperiod cycle, settlement at higher turbidity levels was, however, hindered possibly due to deposition of a fine layer of material on the inside of the bottles. This sediment may have caused the larvae to delay attachment until they found an acceptable place to attach (Havenhand and Svane 1991; McHenry 2005; Svane and Young 1989), ideally one where there is less chance of sedimentation (Prendergast 2010). Because tunicate larvae do not feed, the viability of larvae decreased with time. Others have suggested high levels of turbidity affect locomotor behaviour (coordination of the motions; McHenry 2005) and may also affect sensory structures needed for competent swimming behaviour as well as the orientation process required during settlement on a substrate (Manríquez and Castilla 2007; McHenry 2005; Prendergast 2010; Svane and Young 1989). Lastly, simple physical abrasion and physiological stress generated by an increased level of sediments (Sutherland 2006) could influence the settlement process.

There are several possible explanations for the unattached but metamorphosed individuals that were also observed in the bottles. The larvae may perceive the water surface itself as a substrate for attachment (Hendrickson et al. 2004), which would explain the high incidence in the 0 NTU treatment. In addition, the hydrodynamic conditions within the bottles may not have been ideal to allow for attachment (Carver et al. 2006; Manríquez and Castilla 2007). While there would seem to be adequate substrates present such as aquaculture structures in Orwell Bay, it is possible that these surfaces are covered by a thin layer of sediment. This layer could be an obstacle for tunicate attachment (Kluijver and Leewis 1994; Rocha et al. 1999) or delay attachment until an ideal surface is present (Havenhand and Svane 1991; Marshall and Keough 2003; Osman 1977).

However, even if larval settlement occurs, our results suggest a reduction in juvenile survival with increasing turbidity levels and prolonged exposure. Several hypotheses to explain the reduction of survival under conditions of high turbidity can be proposed based on studies

conducted on adult tunicates (Fiala-Médioni 1979; Petersen and Riisgård 1992; Petersen 2007; Robbins 1983; Robbins 1985a, b; Sigsgaard et al. 2003). Because *C. intestinalis* does not discriminate between inorganic and organic particles during filtration (Robbins 1983; Robbins 1985a, b), and is characterized as a non-stop suspension filter feeder (Carver et al. 2006; Fiala-Médoni 1979), low food quality or high inorganic matter can have important effects (Robbins 1985a; Sigsgaard et al. 2003). When large amounts of inorganic matter are ingested, the animal can have the physically sense of satiation despite a reduced amount of food being ingested (Robbins 1983; Robbins 1985a, b; Petersen 2007). Increasing concentrations of inorganic matter can also cause a gradual decline of the individual's filtration rate (Fiala-Médioni 1979; Petersen and Riisgård 1992; Robbins 1983; Robbins 1984; Robbins 1985a, b; Sisgsgaard et al. 2003). The reduced intake of energy, along with reduced efficiency of nutrient assimilation, could reduce growth and even survival if juveniles follow the same pattern as demonstrated in adult *C. intestinalis* (Petersen and Riisgård 1992; Robbins 1985a). In the event that some juveniles might survive into adulthood in a turbid environment, sexual maturation and output of gametes could also be reduced, which could have a detrimental effect on the establishment of the species.

Variations of turbidity levels in the juvenile survival trial was not considered problematic because it more closely mimicked the variations observed in the natural aquatic environment caused by tides, currents, and wind. Juvenile survival decreased over time in all treatments including the 0 NTU treatment, which should have been ideal environmental conditions for survival and growth of juveniles (based on Cirino et al. 2002). One explanation for the similar mortality rates in all treatments could be due to trapping and accumulation of small air bubbles by the Petri dishes, which was observed at the time of sampling. The air bubbles were presumably being introduced into the tanks from turbulence created at the air water interface from the water returning from the pump and then accumulating under the Petri dishes. Documented negative effects of exposure to air bubbles on marine organisms include reduced growth, decreased the metabolic rate, and possibly direct mortality (Bayne et al. 1976; Davies et al. 2009; Menge et al. 2002). Unfortunately, the accumulation of air bubbles was not quantified during this experiment; therefore, we cannot comment on possible interactions between the air bubbles and the treatments.

Although *C. intestinalis* is known to live in a wide range of environmental conditions (Dybern 1967; Naranjo et al. 1996; Bellas et al. 2003; Carver et al. 2006), this species can be extremely sensitive to some types of perturbations (Petersen 2007). Turbidity levels in an aquatic system can vary considerably over time due to strong tidal cycles and current, high water flow, heavy precipitations, high winds, low bathymetry (Huang et al. 2003), or high rates of soil erosion in surrounding watershed (Marsh 2005; Randhir and Haws 2009; Windom and Stickney 1976). Many of these environmental conditions were observed in Orwell Bay during the summer of 2010 (McLaughlin, unpubl. data). In contrast, the Brudenell-Montague complex, which has a well-established tunicate population showed much lower turbidity levels (McLaughlin, unpublished data). While average turbidity was 7-times higher in Orwell Bay, the inorganic to organic matter ratio was similar to that of the Brudenell-Montague complex (76.8% vs. 73.4% inorganic matter). However, chlorophyll *a* concentrations are approximately 2.8 times higher on average in the Brudenell-Montague complex (McLaughlin, unpublished data), which suggests a greater supply of food. In this study, we only tested the effect of increasing the ratio of inorganic to organic matter (i.e., food concentration was held constant). A logical next step would be to evaluate how changing both the supply of food and turbidity level affects development and survival of *C. intestinalis,* making the results applicable to a wider range of locations.

Because this was a laboratory study, relating our results to aquatic environments such as Orwell Bay must be made with caution. For example, the sediments used in the laboratory trials were not identical to those found in suspension in Orwell Bay; therefore, a different composition of suspended matter could potentially influence the outcome. However, our results do concur with observations of the natural turbid environment of Orwell Bay where there was a lack of tunicate establishment and a high turbidity level (McLaughlin, unpublished data).

Conclusion

This research demonstrated that turbidity, in terms of increased suspended inorganic matter, had a significant negative effect on fertilization, settlement, and juvenile survival of *C. intestinalis*.

Although increased turbidity did have a negative impact on *C. intestinalis*, it did not completely eliminate any of these processes. Therefore, in a turbid environment it would be possible to have tunicate gametes meet, fertilize, develop to the larval stage, settle and potentially survive into adulthood. Given the fact that tunicate establishment in Orwell Bay has been unsuccessful despite multiple introductions, turbidity could be one of the factors limiting the establishment of *C. intestinalis* and perhaps other tunicate species. The results of this study provide insight into one environmental factor which can play important roles in the establishment of tunicate species, such as *C. intestinalis,* and in identifying other locations that are at risk of successful invasion from tunicate species.

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