Very Low Concentrations of Maritime Exhaust Gas Cleaning System Effluent Impair Fertilization and Larval Development in the Green Sea

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Very Low Concentrations of Maritime Exhaust Gas Cleaning System Effluent Impair Fertilization and Larval Development in the Green Sea Urchin *Strongylocentrotus droebachiensis*

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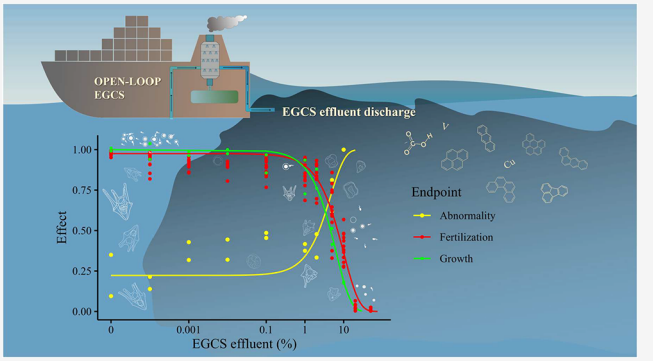
ABSTRACT: Exhaust gas cleaning systems (EGCS) are increasingly used to meet IMO sulfur regulations while permitting the continued use of heavy fuel oil. EGCS effluents are acidic and contain metals, polycyclic aromatic compounds, and other contaminants that are known to affect marine organisms. Whole effluent toxicity tests were conducted on fertilization and larval development of the green sea urchin *Strongylocentrotus droebachiensis*using a 0−10% dilution series of open-loop EGCS effluent. Fertilization success was significantly reduced at the lowest concentration tested (0.0001%). Larval abnormalities increased with effluent concentration, reaching 100% at 10% effluent, where larvae failed to develop skeletons and reach the pluteus stage. Larval growth rates declined with increasing exposure and ceased at a 10% effluent. Exposure to the lowest test concentration caused an estimated additional mortality of 4.6% day^{-1}, indicating a high risk of population-level impacts. As carbonate chemistry remained unchanged below 0.1% effluent, the toxicity was attributed primarily to contaminants. These findings indicate that EGCS effluents pose a significant threat to marine life.



KEYWORDS: *sea urchin, larval development, polycyclic aromatic compounds, alkylated PAH, exhaust gas cleaning system effluent, shipping emission*

1. INTRODUCTION

Ships using heavy fuel oil (HFO) emit exhaust containing a range of compounds that have negative impacts on the environment, including carbon dioxide (CO_2), polycyclic aromatic compounds (PACs), sulfur oxides (SO_x), nitrogen oxides (NO_x), combustion particles, heavy metals, and ozone (O_3).[1−5] To mitigate airborne pollution and associated negative effects on human health, a global limit of 0.5% sulfur content in shipping fuels was implemented in January 2020 by the International Maritime Organization (IMO).[6] In areas classified by the IMO as sulfur emission control areas (SECAs), the maximum allowed sulfur content in shipping fuel was set to 0.1% already in 2015.[7] The main aim of this "sulfur cap" was to limit atmospheric emissions of SO_x and particulate matter (PM) from ships. Vessels with combustion engines can achieve compliance either by using low-sulfur fuel alternatives such as marine gas oil (MGO), hybrid fuels such as very low sulfur fuel oils (VLSFO, <0.05% sulfur) or ultra low sulfur fuel oils (ULSFO, <0.01% sulfur), or by installing an exhaust gas cleaning system (EGCS, also called scrubber system) while continuing to use cheaper residual fuels (e.g., HFO) with a high sulfur content. Scrubber systems are designed to capture SO_x from the exhaust by leading it through a fine heated spray of seawater or a mixed alkaline solution. At the same time, metals, PACs, nitrogen compounds, and combustion particles in the exhaust also dissolve in the wash water, producing a highly contaminated EGCS effluent. EGCSs exist in the form of open-loop (OL) designs, where seawater is pumped into the EGCS and subsequently released into the sea often without any cleaning steps, closed-loop (CL) designs, where an alkaline solution is recirculated within the EGCS and a much smaller volume of effluent (albeit with higher contaminant loads) is released as bleed-off and when the circulating cleaning solution is replenished, and hybrid systems which can alternate between OL and CL modes.[8] CL systems often have some type of effluent-cleaning steps to remove particulates. CL systems need to constantly release smaller amounts of concentrated effluent (called bleed-off) while particulates are separated and collected as sludge. The sludge is stored onboard until it can be disposed off at ports with appropriate reception facilities. Discharge rates can vary greatly, especially for OL systems,

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depending on the physical and chemical properties of the water being used in the EGCS and the targeted SOx removal rate.[9] Discharge rates also depend on the current engine load.[10] For modeling purposes, Jalkanen et al.[11] assumed EGCS discharge volumes of 45 m^3 per MWh engine power for OL systems, and 0.3 m^3 MWh^{-1} for CL systems, respectively. Teuchies et al.[12] measured discharge volume ranges of 36.7−227.1 m^3 MWh^{-1} (OL) and 0.22−0.88 m^3 MWh^{-1} (CL). For a medium-sized vessel with a 12-MW engine, this amounts to 10569.6−65404.8 m^3 (OL) and 63.36−253.44 m^3 (CL) per day, assuming 24 h of operation and 100% EGCS utilization rate.

IMO's Global Integrated Shipping Information System (GISIS) listed 671 ports with EGCS effluent reception facilities in accordance with MARPOL Annex VI (as of February 2023).[13] However, 82% of 5080 registered EGCSs are of the OL type.[14] The number of installed EGCSs s estimated to increase to 5299 by 2026, due to the economic advantage of continued use of HFO in combination with an EGCS as an alternative to switching to low-sulfur fuels.[15] However, simulations of the total global fleet equipped with EGCSs, applying actual fuel costs and other expenses associated with the installation and use of the equipment, show that >95% of the ships with the most common EGCS systems reach the point of economic breakeven within five years.[16]

While the sulfur cap was designed to reduce atmospheric sulfur emissions to improve air quality in populated areas, it opened a new pollution pathway to the marine environment when allowing the use of EGCSs. There is an urgent need to understand and address the biological impacts of the continuous release of extensive amounts of EGCS effluent, both in terms of localized high-emission contexts (i.e., shipping lanes) and in relation to general increased pollution levels in the marine environment. This potential impact was addressed already before the legislation came into action.[17] OL-EGCS effluent is identified as the single largest pollution source stemming from ship waste streams.[18] Recent chemical screenings of EGCS effluents have shown alkylated polycyclic aromatic hydrocarbons (alkyl-PAHs) to dominate the PAC content of EGCS effluents.[2,19,20] The alkylated PAHs are a large group of PACs, present in EGCS effluent along with other substituted PAHs. These compounds are known to be toxic, sometimes even more toxic than their parent compounds, but, they are rarely measured and exotoxicological threshold values for marine organisms are lacking. Complex combined effects of PAH mixtures and metals have been documented in marine organisms (e.g., Barata et al.[21] and Nogueira et al.[22]), including for EGCS effluents.[23] Because of suspected mixture toxicity effects, it is important that whole effluent toxicity (WET) tests are performed for results to be ecologically relevant and to support solid ecological risk assessment.[24,25] EGCS effluents are released to the surface water, and organisms present in the upper pelagic will therefore be immediately affected by the discharges. The upper pelagic harbors its own ecosystem, including larvae of both pelagic and benthic species. Most marine fish and invertebrate species have pelagic reproduction stages and/or pelagic larval development[26] and since early life stages generally are more sensitive to pollution than the adults, organisms of the whole coastal marine ecosystem are at risk.[27−29]

The aim of this study was to quantify the effects of EGCS effluent on the fertilization and early development of the green sea urchin (*Strongylocentrotus droebachiensis*), targeting sensitive endpoints and conducting WET tests and experiments with high environmental relevance. CRED (Criteria for Reporting and Evaluating Ecotoxicity Data) methodology[30] was applied to generate scientific data also fit for regulatory purposes in accordance with EU[25] and IMO guidelines.[24]

2. MATERIALS AND METHODS

2.1. EGCS Effluent Sampling and Preparation

The EGCS effluent was collected from the outlet of an OL-operated (STI Marine, Seoul, South Korea) container ship LEO C (Danaos Shipping Co. Ltd.) while in the English Channel (51° 8.477′N 1° 36.246′ E; engine 21.7 MW; specific fuel consumption 4010 kg h^{-1}; service speed 18 knots; engine load 33.5%; scrubber model STI-SCR-O-401101) on 16 November 2021 on its journey from Antwerp (Belgium) to Gebse (Turkey). At the time of collection, the ship was running on HFO (sulfur content ∼2.5%). The effluent was collected from the outlet pipe into 5 L acid-acetone rinsed glass jars (1.75% hydrochloric acid analytical grade, acetone 99% purity, both from Penta Chemical, Prague), filled to the brim, sealed airtight, and stored dark and cold (+4 °C) onboard until further transfer by refrigerated transport (dark at +5 °C) to Kristineberg Marine Research Station, Sweden. Samples were simultaneously collected for PAC analysis into ultrapure 1 L amber glass bottles, and for metal analysis into 50 mL acidified polyethene tubes (V, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Cd, Pb, U) and 40 mL amber glass vials (Hg), stored dark and cold (+4 °C) and shipped within a week for analysis. Values of metal concentrations reported here originate from samples collected at the sampling station located closest to the EGCS effluent sampling point during the onboard sampling campaign. These samples were used to replace broken bottles for metal analysis from the original sampling point. This sampling station was also located in the English Channel (48° 87.18′ N, 3° 52.59′ W; engine 21.84 MW; specific fuel consumption 4020 kg h^{-1}; engine load 37%) and the same fuel was used. Analytical methods are described in detail in Genitsaris et al.[31]

At Kristineberg Marine Research Station, the effluent samples were stored under cool (+4°C) and dark conditions until use. Only equipment made of glass or metal was used in the preparation of solutions and in the experiment. Metal and glassware were either heated in a muffle furnace (+550 °C, 4 h) or rinsed sequentially with 0.1 M HCl, five times with Milli-Q (0.2 μm filtered), and acetone (ultrapure analytical grade 99.5%, Thermo Scientific Chemicals) to remove traces of metals, organic matter, and organic contaminants prior to use. All equipment was covered with muffled (+550 °C, 4 h) aluminum foil. Dilutions of effluent water were prepared using filtered surface seawater (FSW) (0.5 μm, 26.8 ± 2.3 PSU, mean ± SD) collected at a depth of 6 m in the Gullmarfjord, Sweden. The same FSW source was used throughout the experiment. Glass pipettes and volumetric flasks were used for dilution of the EGCS effluent to achieve precise concentrations. The low concentration range was achieved using a stepwise serial dilution technique, which maintains a set dilution factor for homogeneous dissolution of contaminants (SM Table S1).

EGCS effluents are acidic due to the sulfuric acid component and thus affect the seawater carbonate chemistry. In the stock seawater used for all treatments, two parameters of the carbonate system, pH and total alkalinity (TA), were measured. pH was monitored using a Metrohm (827 pH lab) electrode calibrated on the total scale (pH_T) using TRIS (Tris/HCl) buffer solutions with a salinity of 28 (provided by the Unité d'Océanographie Chimique, Université de Liège, Belgium). TA was measured on filtered samples by automatic titration (Titroline α plus, SI Analytics), following recommendations by Dickson et al.[32] Other parameters of the carbonate system (pCO2, ΩCa, and ΩAr) were calculated from a salinity of 28, measured temperature, pH_T, and TA, following recommendations by Dickson et al.[32] (SM Table S2).

2.2. Sea Urchins Sampling and Artificial Spawning

About 100 adult *S. droebachiensis* were collected by divers in the vicinity of Droebak in the Oslofjord, Norway, in February 2021. The sea urchins were transported within 4 h in aerated FSW (32 PSU, + 4 °C) to the Kristineberg Marine Research Station. All animal experiments were conducted in accordance with the Swedish national animal welfare legislation (SFS 2018:1192) and approved as required by the Swedish

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Environ. Sci. Technol. 2026, 60, 5231−5241

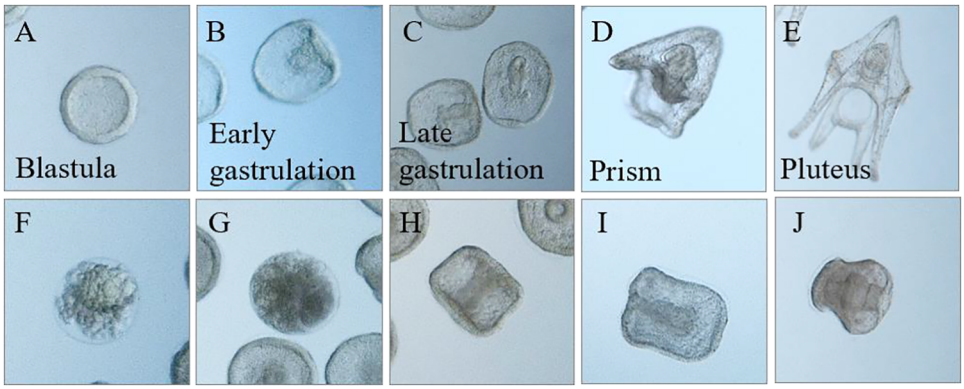

Figure 1. Photographic representation of normal (panels A–E) and abnormal (panels F–J) development of green sea urchin (*S. droebachiensis*) larvae from selected EGCS effluent treatments. Images are not displayed according to scale but to show distinguishing features.

Board of Agriculture's Experimental Animal Unit (Ethics Approval Numbers: Dnr 5.2,18–01447/2022 and Dnr 5.2.18–01448/2022). The sea urchins were maintained in flow-through aquaria with deep seawater under ambient conditions and fed *ad libitum* with sugar kelp (*Saccharina latissima*) until use. In April 2022, a randomized subset of adult *S. droebachiensis* were selected, and artificial spawning was induced via intracelomic injection of 0.5 M potassium chloride (KCl) in FSW. The eggs were collected in FSW, and sperm were collected dry and kept in a 1.5 mL Eppendorf tube on ice until use.

2.3. Fertilization Experiments

Fertilization experiments were conducted in 2 mL muffled (+500 °C, 4 h) broad-bottom glass cups. 100 μL of a solution of *S. droebachiensis* eggs (1 egg μL^{-1} FSW) and 20 μL of mixed sperm solution (diluted 1:1000 in FSW) were added to fertilization cups (1.5 mL of FSW; 10%, 5, 2, 1, 0.1, 0.01, 0.001, 0.0001 and 0% of EGCS effluent in FSW) with 6 replicates per treatment. Detailed numbers of eggs and viability were documented and provided in the compiled data file. The experiment was carried out in darkness at a constant temperature (around +11 °C). The fertilization process was stopped after 15 min by adding a drop of paraformaldehyde solution (4% in FSW). The total number of fertilized and unfertilized eggs was counted under a stereomicroscope (Leica MZ FLIII). All samples were counted by two scientists independently to ensure accuracy. A second fertilization experiment was performed using the same protocol with two additional treatment concentrations (20 and 50% EGCS effluent) to obtain a full dose–response curve spanning the entire effect range of 0 to 100% impact. In each experiment, sperm from one male and eggs from one female sea urchin were used. Several individuals were tested to determine egg-sperm compatibility, and only combinations of individuals producing viable zygotes were selected for the experiment.

2.4. Larval Development Experiment

Eggs from each female were concentrated into a 1 L glass beaker (1.3 million eggs) and fertilized by the addition of 25 μL of dry sperm from compatible males according to Dorey et al.[33] A density of 4 larvae per mL was used, limiting the impact of larvae on the seawater physicochemistry and thus ensuring high survival and normal larval development in control conditions. Fertilized eggs were transferred and exposed in 300 mL glass flasks with screw caps (n = 2 per treatment) filled with FSW mixed with different concentrations of EGCS effluent previously prepared (Section 2.1) (10, 5, 2, 1, 0.1, 0.01, 0.001, 0.0001, and 0% in FSW). The experiments were carried out in a constant temperature room (+11 °C) in darkness. Once the larvae had developed a functional stomach (6 days post-fertilization (dpf)), they were fed daily with a suspension of microalgae (*Rhodomonas sp.*) at a concentration of 3000–6000 cells mL^{-1} from an axenic culture. Previous work showed that the addition of such a small amount of *Rhodomonas* culture had no effect on the water chemistry or the normal development of the larvae.[33] A 10 mL subsample from each flask was collected daily for a total of 10 days to assess abnormality and larval development.[34] The collected subsamples were fixed with 0.2 mL of paraformaldehyde (4% in FSW). In each subsample, all larvae were counted and scored as normal or abnormal. Abnormal larvae at 1 dpf were those that had uneven cell division and no visible blastula formation (Figure 1F). At 2 dpf, abnormal larvae would have darkened primary mesenchyme cells and the absence of gut as well as darkened blastocoel (Figure 1G). At 3 dpf (gastrulation), when gut formation should commence, larvae were scored as abnormal if they remained in the blastula stage with a darkened blastocoel or showed abnormal gut development (Figure 1H). From 4 to 10 dpf, larvae were supposed to change into pluteus (Figure 1E). Larvae that failed to develop arms, a functional skeleton, or a mouth (Figure 1I,J) were scored as abnormal. Approximately 10 photographed larvae were randomly chosen for subsequent morphometric measurements using ImageJ. Measured variables included body length (BL), left and right body rod length (BRL), posterolateral arm length (PLL), and postoral rod length (POL)[35] (SM Figure S1). The length per pixel (μm) from the microscopic images was previously determined using a micrometric ruler. A conversion factor was used for converting the raw data (length in the number of pixels) measured using ImageJ to μm.

2.5. Calculations and Statistical Analyses

Statistical analysis was carried out using R (v. 4.4.0)[36] in RStudio (v. 2023.12.1 Build 402). Fertilization success was calculated as the percentage of eggs successfully fertilized. The data from the two fertilization experiments were pooled for further analysis following a nonsignificant difference between experimental runs (PERMANOVA with EGCS effluent concentration and experimental round as fixed factors, $F_{1,87}$ = 2.9354, p = 0.0936) as well as no significant interaction between experiments and concentration ($F_{7,95}$ = 1.3202, p = 0.2554). Larval abnormality (in %) was calculated as the proportion of abnormal larvae to total larvae observed at 10 dpf. This time point was chosen because EC calculations assume a sigmoidal dose–response relationship at a specific time. 10 dpf was chosen to best capture persistent and morphologically distinct abnormalities in fully formed pluteus larvae. The somatic growth rate was measured as the increase in body length over time (μm day^{-1}) and calculated as the coefficient of the significant logarithmic relationship between the body length (μm) and time (day). Allometries (μm arm segment μm^{-1} of organism) were calculated for the BRL, PLL, and POL as the coefficient of the significant linear relationship between the longest of the two arms (left and right) and the body length.

Since fertilization data was available at a sample size of n = 12 per treatment (except for 3 concentrations (0.01, 20, and 50%) which had only n = 6), the lowest observed effect concentration (LOEC) and the no observed effect concentration (NOEC) values were calculated using a 1-factor PERMANOVA with 10,000 permutations using the R package vegan (v. 2.6–6.1).[37] Pairwise comparison was used to establish NOEC/LOEC values using the Bonferroni method for correcting for multiple comparisons.

A dose–response model was fitted to the data to obtain 10 and 50% effect concentrations (EC_{10} and EC_{50}, respectively) using the R package

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Environ. Sci. Technol. 2026, 60, 5231–5241

Table 1. Concentrations (ng L^{-1}) of Polycyclic Aromatic Compounds (PACs) in the EGCS Effluent[a,b]

Compound	Total (ng L^{-1})	Dissolved (ng L^{-1})	Particle associated (ng L^{-1})	Particle associated (%)	Number of rings
Naphthalene	8958	6890	2068	23	2
2-methylnaphthalene	4969	3886	1083	22	2
1-methylnaphthalene	4521	3705	816	18	2
C2-alkylnaphthalene	7541	5942	1599	21	2
C3-alkylnaphthalene	4426	2701	1726	39	2
C4-alkylnaphthalene	1407	623	785	56	2
Acenaphthylene	67.6	55.2	12.5	18	2.5
Acenaphthene	387	355	32	8	2.5
Fluorene	1135	943	192	17	2.5
C1-alkylfluorene	2139	1422	717	34	2.5
C2-alkylfluorene	1243	689	554	45	2.5
Phenanthrene	4499	3500	999	22	3
C1-alkylphenanthrene	4644	2685	1959	42	3
C2-alkylphenanthrene	2869	971	1898	66	3
C3-alkylphenanthrene	1089	167	922	85	3
C4-alkylphenanthrene	640	68.3	571	89	3
Anthracene	<LOD (1.67)	<LOD (1.67)	<LOD (1.67)	n/a	3
Fluoranthene	195	141	55	28	3.5
Pyrene	475	195	280	59	4
C1-alkylfluoranthene/pyrene	629	241	388	62	3.5/4
Benzo[a]anthracene	18.4	<LOQ (3.80)	18.4	100	4
Chrysene	214	49.8	164	77	4
Benzo[b]fluoranthene	26.7	<LOQ (3.77)	26.7	100	4.5
Benzo[k]fluoranthene	9.2	<LOQ (2.51)	9.2	100	4.5
Benzo[a]pyrene	<LOD (3.70)	<LOD (3.70)	<LOD (1.67)	n/a	5
Indeno[1,2,3-cd]pyrene	<LOD (1.67)	<LOD (1.67)	<LOD (1.67)	n/a	5.5
Dibenzo[a,h]anthracene	29.4	<LOD (3.43)	29.4	100	5
Benzo[g,h,i]perylene	175	<LOD (3.43)	175	100	6
Benzo[c]phenanthrene	<LOD (0.33)	<LOD (0.33)	<LOD (1.67)	n/a	4
∑16 PAH	16189	12129	4061		
∑12 alkylated PAH	36117	23100	13018		
∑29 PAC	52306	35229	17079		

[a]Concentrations are measured as total, dissolved in the effluent, and particle associated PACs. The percentage of total PACs associated with particles is also provided. Number of rings indicates the number of 6-carbon aromatic rings composing the compound. Half rings indicate 5-carbon rings. [b]n/a = not applicable. LOD indicates the minimum level of detection. Red filling indicates that more than half of the concentration of the specific compound is found associated with combustion particles.

drc (v. 3.0−1), as described by Ritz et al.[38] As fertilization success and growth rate were expected to decrease with increasing EGCS effluent concentrations, the upper limit of the dose−response model for each parameter was fixed to the arithmetic mean of the dependent variable in the control treatment, while the lower limit was fixed to zero, as both variables were assumed to be positive values. Abnormality was expected to increase with the EGCS effluent concentration, and the lower limit of the dose−response model was fixed to the arithmetic mean of the proportion of deformed larvae in the control treatment. The best-fitting model was selected using the Akaike information criterion (AIC).[39] The selection of the model was refined by excluding negative EGCS effluent concentrations and excluded negative dependent variables. In addition, for the variables of abnormality and fertilization success, the pool was restricted to models bound to output values bounded by [0, 1] (i.e., $0 \leq y \leq 1$).

3. RESULTS

3.1. Chemical Composition of the EGCS Effluent

The EGCS effluent was analyzed for 17 PAHs (the traditional 16 US EPA PAHs[40] and benzo[*c*]phenanthrene) and 12 alkylated PAH homologues[40] (Table 1). The effluent was also analyzed for several metals (Table 2) and for the presence of combustion particles.

Of the PACs, naphthalene (8958 ng L^{-1}) and C2-alkylnaphthalene (7541 ng L^{-1}) occurred at the highest concentrations, followed by C1-alkylphenanthrene (4644 ng L^{-1}) and phenanthrene (4499 ng L^{-1}). The relative concentrations of fluorene, phenanthrene, and fluoranthene/pyrene and their corresponding alkylated homologues displayed bell-

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Table 2. Concentrations (Mean ± SD) of Metals in the EGCS Effluent

	EGCS effluent ($\mu g\ L^{-1}$)	LOD ($\mu g\ L^{-1}$)	LOQ ($\mu g\ L^{-1}$)
Vanadium(V)	249.7 ± 1.7	0.005	0.015
Chromium (Cr)	26.0 ± 0.5	0.001	0.03
Manganese (Mn)	1.26 ± 0.03	0.005	0.015
Iron (Fe)	112.0 ± 3.1	0.01	0.03
Cobalt (Co)	0.527 ± 0.01	0.0001	0.0003
Nickel (Ni)	69.4 ± 1.7	0.001	0.003
Copper (Cu)	1.593 ± 0.121	0.002	0.006
Zinc (Zn)	94.8 ± 3.6	0.005	0.015
Arsenic (As)	1.85 ± 0.05	0.02	0.06
Cadmium (Cd)	0.031 ± 0.0016	0.0001	0.0003
Mercury (Hg)	0.0011 ± 0.0002	0.0001	0.0003
Lead (Pb)	0.1677 ± 0.0011	0.001	0.003
Uranium (U)	6.57 ± 0.22	0.001	0.003

shaped patterns, while the naphthalene group showed a skewed distribution with nonalkylated naphthalene present at the highest concentration (Table 1). The total concentration of alkylated PACs ($\sum$12 alkylated PACs) (36118 ng L^{-1}) was more than double (2.23 times) the concentration of the 16 US EPA PAHs (16188 ng L^{-1}). PACs composed of more than four benzene rings were mostly (59−100%) associated with the particulate fraction, i.e., combustion particles. This was also true for the two and three-ringed alkylated PAHs C4-alkylnaphthalene (56%), C2-alkylphenanthrene (66%), C3-alkylphenanthrene (85%), and C4-alkylphenanthrene (89%). The 2−3.5 ringed PAHs naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, and fluoranthene were mostly (72−92%) present in the dissolved fraction of the EGCS effluent, while the solubility decreased with the degree of alkylation within each compound group. Anthracene concentrations were below detection (LOD = 1.67 ng L^{-1}).

The highest individual metal concentrations of the EGCS effluent were attributed to the metals V (256.87 ± 6.74 $\mu g\ L^{-1}$, mean ± SD), Fe (123.30 ± 9.27 $\mu g\ L^{-1}$), Zn (117.17 ± 16.23 $\mu g\ L^{-1}$), Ni (72.53 ± 2.53 $\mu g\ L^{-1}$), Cr (26.57 ± 4.87 $\mu g\ L^{-1}$), and Cu (23.56 ± 28.08 $\mu g\ L^{-1}$) (Table 2). The concentration of combustion particles in the EGCS effluent was 1.43 × 10^6 particles L^{-1} with a Feret diameter of 20 ± 10 μm (mean ± SD) and perimeter of 140 ± 194 μm (mean ± SD).[41]

pH_T, TA, and saturation state (Ω) of calcite and aragonite declined with increasing EGCS effluent concentration. The pH_T is 2500 in fully saline oceanic water; however, the salinity of the seawater used in this study was 26.8 ± 2.3 PSU (mean ± SD) and thus not fully saline. This was indicated also by a slightly lower pH_T. When $\Omega < 1$, the seawater is considered corrosive for organisms building shells or supporting structures from calcite or aragonite. There was a sudden drop in pH_T at an EGCS effluent concentration between 0.1 to 1% and seawater became corrosive in terms of calcite and aragonite availability at an EGCS effluent concentration above 2% (SM Table S2).

3.2. Fertilization Success

There was a statistically significant reduction in the fertilization success at the lowest treatment concentration (0.0001% EGCS effluent equivalent to 1 μL L^{-1} EGCS and a $\sum$29 PAC of 5230 ng L^{-1}) (1-factor PERMANOVA: $F_{1,23}$ = 8.947, adj p = 0.012). The fertilization success in the control treatment was 97.57 ± 1.22% (mean ± SD), and it was reduced by almost 4.5% to 93.09 ± 5.04% (mean ± SD) at 0.0001% EGCS effluent exposure just after 15 min of exposure (SM Table S3). As the LOEC was the lowest concentration tested (0.0001% EGCS effluent), a NOEC could not be established but will naturally lie between 0 and 0.0001% EGCS effluent concentration (Table 3). The best-fitting dose−response model was a three-parameter Weibull function (W1.3 in R, package drc) as in eq 1

$$f(x) = 0 + (d - 0)\exp(-\exp(b(\log(x) - e))) \quad (1)$$

where x is the EGCS effluent concentration. Parameter d (the upper limit of the dose−response curve) was fixed to the mean proportion of fertilized larvae in the control treatment, d = 0.9757, and parameters b and e are fitted model parameters (b = 1.0913 ± 0.080695; e = 10.1183 ± 0.482 (mean ± SE)). From the fitted model, the EC_{10} concentration was predicted to be 1.287 ± 0.1826% (mean ± SE) of the EGCS effluent in FSW, and the EC_{50} concentration was predicted to be 7.2319 ± 0.3242% (mean ± SE) of the EGCS effluent in FSW (Figure 2A and Table 3). The effect concentrations are expressed as a percentage dilution where undiluted EGCS is 100%. Percentages are chosen over concentrations of individual compounds or sums of compounds since the effluent consists of a complex mixture of known and unknown compounds, which is tested as an entity using WET test methodology.[42]

3.3. Larval Abnormality

Abnormal larval development was observed from 1 to 10 dpf. The abnormalities resulting from EGCS exposure were in the form of lysed cells, irregular cell division, unclear gastrulation, absence of skeleton, and uneven arm lengths (Figures 3 and SM S2). For estimating the EC values, the best-fitting model was a three-parameter Weibull function (function W2.3u in R, package drc) as in eq 2

$$f(x) = c + (1 - c)\exp(-\exp(b(\log(x) - e))) \quad (2)$$

where x is the EGCS effluent concentration. Parameter c (the lower limit of the dose−response curve) was fixed to the arithmetic mean of the proportion of abnormal larvae in the control treatment; c = 0.2226, b, and e are fitted model parameters (b = 1.174 ± 0.4826 (mean ± SE); e = 4.6283 ± 0.9397) (mean ± SE). From the fitted model, the EC_{10} concentration was predicted to be 0.6807 ± 0.5481% (mean ± SE) EGCS effluent in FSW, and the EC_{50} to 3.3872 ±

Table 3. Summary of Tested Effects (NOEC and LOEC) and Modeled Effects (EC_{10} and EC_{50} (Mean ± SE)) of EGCS Effluent on Early Life Stages of *S. droebachiensis*; Concentrations Are Expressed as a Percentage of EGCS Effluent in the Exposure Water[a,b]

Endpoint	NOEC	LOEC	EC_{10}	EC_{50}
fertilization success (%)	<0.0001	0.0001	1.287 ± 0.183	7.232 ± 0.324
larval abnormality (%)	n/a[b]	n/a[b]	0.681 ± 0.548	3.387 ± 0.798
larval growth (μm ln(day)$^{-1}$)	n/a[b]	n/a[b]	0.884 ± 0.223	4.621 ± 0.372
allometry of POL, PLL, BRL[a] (μm μm^{-1})	n/a[b]	n/a[b]	n/a[b]	n/a[b]

[a]POL = postoral rod length, PLL = posterolateral arm length, and BRL = body rod length. [b]n/a = not applicable.

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Environ. Sci. Technol. 2026, 60, 5231−5241

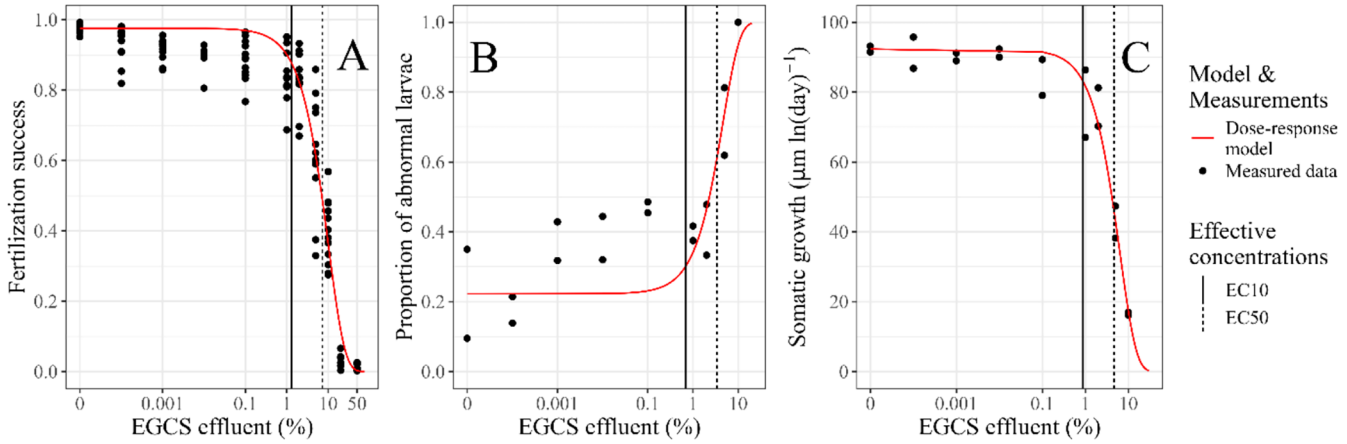

Figure 2. Dose−response curve fitting was performed to derive $EC_{10/50}$ estimations on fertilization success (A), larval abnormality (B), and somatic growth (C).

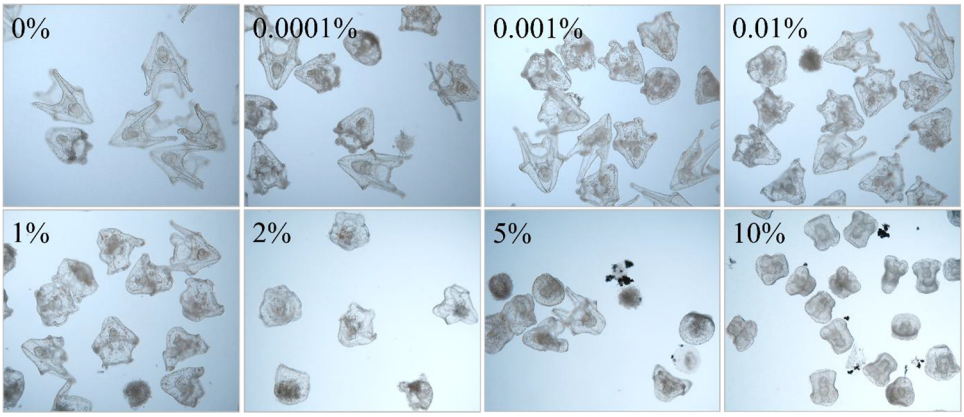

Figure 3. Larval development, growth, and allometries are affected, increasing with increasing concentration of EGCS effluent. Images are not displayed according to scale, but to show distinguishing features.

0.7976% (mean ± SE) EGCS effluent in FSW (Figure 2B and Table 3).

3.4. Growth

There was a negative relationship between the EGCS effluent concentration and larval size. The best-fitting model for somatic growth was a three-parameter Weibull function (W1.3 in R, package drc) as in eq 1, where x is the EGCS effluent concentration (% in FSW). Parameter d (the upper limit of the dose−response curve) was fixed to the mean growth of larvae in the control treatment, $d = 92.327\ \mu m\ ln(day)^{-1}$, and parameters b and e are fitted model parameters ($b = 1.1389 \pm 0.1529$ (mean ± SE); $e = 6.3757 \pm 0.5244$ (mean ± SE)). From the fitted model, the EC_{10} was predicted to be 0.8839 ± 0.2235% (mean ± SE) EGCS effluent, and the EC_{50} to be 4.6213 ± 0.3720% (mean ± SE) EGCS effluent (Figure 2C and Table 3).

3.5. Allometries

Most of the larvae exposed to 5 and 10% EGCS effluent in FSW did not develop skeletons, which resulted in a small number of data points available for analysis at higher concentrations (SM Figure S3). Therefore, the 5 and 10% effluent concentrations were excluded from further analysis (SM Figure S4). Linear regression analyses performed on the remaining data showed that EGCS effluent exposure had a significant effect on the BRL allometry (adj. $R^2 = 0.232$, $p = 0.047$) but no significant effects on the allometries for PLL (adj. $R^2 = -0.04544$, $p = 0.5221$) and POL (adj. $R^2 = -0.0536$, $p = 0.5714$) (SM Figure S4). We consider this endpoint unfit for testing the effects of complex oily mixtures.

4. DISCUSSION

Because adult individuals of the green sea urchin (*S. droebachiensis*) are benthic, the risk of exposure to EGCS effluent is highest during their pelagic fertilization and larval stages. Early life stages of marine organisms are generally more sensitive to pollution than their adults.[27] Kourkoutmani et al.,[43] e.g., observed a higher mortality in invertebrate larvae than in adult zooplankton in general when a plankton community was exposed to EGCS effluent. Fertilization success was the most sensitive of all analyzed endpoints with a statistically significant reduction in the proportion of fertilized eggs observed already at an EGCS effluent concentration of 0.0001% (LOEC), corresponding to a ∑16 PAH concentration of 16.19 pg L^{-1}. The concentration would be equivalent to 1 mL of EGCS effluent in 1 m^3 of seawater. Finding effects at such low concentrations on a vital endpoint such as egg fertilization is alarming, since EGCS effluents are thus likely to have a potential impact on populations of *S. droebachiensis* sea urchins also in areas where effluents are more diluted than in shipping lanes. However, when comparing EC_{10} values, larval development was more sensitive (0.681 ± 0.548% (mean ± SE) EGCS effluent in FSW) than both somatic growth (0.884 ± 0.223% (mean ± SE)

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Environ. Sci. Technol. 2026, 60, 5231−5241

EGCS effluent in FSW) and fertilization success (1.287 ± 0.183% (mean ± SE) EGCS effluent in FSW) (Table 3). There is a difference between endpoint measurements, which should be noted, while NOEC/LOEC are measured concentrations of the EGCS effluent the EC values are concentrations derived from best-fit models from dose−response curves.

EGCS effluent is a complex mixture with a low pH, containing PACs and other oil-related organic compounds, metals, nutrients, combustion particles, and perhaps other unknown contaminants, and further research is needed to fully comprehend how the mixture toxicity is mediated. In our study, the EGCS effluent originated from HFO, which is a common type of shipping fuel.[44] Most studies on EGCS effluent chemistry report the 16 EPA PAHs, or a subset of them, and/or a selection of 5 to 15 metals.[12,20,45,46] The 16 EPA PAHs include only parent PAHs, and in EGCS effluents naphthalene and phenanthrene often occurr at high concentrations followed by pyrene or fluorene.[12,20,45] Only few studies include a broader spectrum of PACs, e.g., alkylated and substituted PAH homologues and heterocyclic PACs.[19,20,23] When alkylated PACs are measured, as in our study, they completely dominate the PAC content of the EGCS effluent, with C0−C4 naphthalene and phenanthrene groups present at the highest concentrations. The ∑16 PAH content can be used as a blunt tool to normalize EGCS effluents to each other, and our effluent had a ∑16 PAH concentration of 16.19 $\mu g\ L^{-1}$, which is similar to 11.0 $\mu g\ L^{-1}$ reported by Thor et al.,[20] while Du et al.[47] and Achten et al.[19] analyzed several different OL-EGCS effluents with ∑16 PAH concentration ranging from 4.1 to 16.0 $\mu g\ L^{-1}$ and from 1.13 to 24.2 $\mu g\ L^{-1}$, respectively. Our effluent was in the higher concentration range and is therefore likely to induce a greater toxic response at higher dilutions. 25% (4061 ng L^{-1}) of the PAHs and 36% (13018 ng L^{-1}) of the alkylated PAHs were found to be associated with combustion particles. PAHs with four or more rings and PAH homologues with a higher degree of alkylation will thus, to a greater extent (<50%), be associated with particles than being dissolved in effluent. The association between PACs and combustion particles is often very strong, and their bioavailability is therefore likely low for both macrofaunal uptake and microbial degradation.[48,49] Exposed *S. deroebachiensis* eggs, sperm, fertilized eggs, and larvae, which do not yet feed (<6 days old), will thus likely be more affected by the dissolved PACs in the effluent, including naphthalene and alkylated C1−C3 homologues, acenaphthylene, acenaphthene, fluorene and alkylated C1−C2 homologues, phenanthrene and C1-homologues, and fluoranthene (Table 1). Based on quantitative structure−activity relationship (QSAR) analysis, alkylated PACs have been suggested to contribute largely to EGCS effluent toxicity,[50] but since the effluent was tested as an entity, it is difficult to determine which compounds were primarily responsible for the observed toxic effects.

The pH of OL-EGCS effluents can range from 3 to 5.6 and is primarily mediated by sulfuric acid.[51,52] We observed a decrease in seawater pH and alkalinity with increasing concentrations of EGCS effluent, with a sudden change in alkalinity around 0.1−1% effluent, corresponding to a pH_T of 7.95−7.87 (SM Table S2). Effects observed at concentrations below 0.1% effluent are thus more likely to be attributed to the contaminants than to changes in the carbonate chemistry. At higher exposure concentrations (above 1−2%), both carbonate chemistry and contaminants will stress the organisms, likely acting in concert. The EC_{50} values for all endpoints ranged between 3.4 and 7.2% EGCS effluent in FSW (Table 3). Since there is a significant linear relationship between the concentration of EGCS effluents and pH_T ($pH_T = -0.1063 \times$ concentration +7.9562; $R^2 = 0.99$), we can calculate the pH_T thresholds for the different measured endpoints when EGCS effluents are released to the marine environment (26.8 ± 2.3 PSU, mean ± SD) as pH_T 7.2 for fertilization success, pH_T 7.46 for somatic growth and pH_T 7.6 for abnormality. These pHs are consistent with previous calculated thresholds for *S. droebachiensis* based on dynamic energy budget theory (pH 7.5 for growth; Jager et al.[53]). Current IMO regulations allow release of EGCS effluent with a pH ≥ 6.5; however, during maneuvering and transit, an even lower pH value is allowed (maximum difference of 2 pH units between the inlet- and overboard discharge waters).[54] According to our results, which are limited to larvae of one species, the pH limit value should be more restrictive, and a value of $pH_T \geq 7.95$ should be used to minimize biological effects in seawater with a high salinity. This threshold should likely be even higher in less saline waters with a lower buffering capacity. The results support recent amendments to the IMO guidelines for risk and impact assessment of the discharge water from EGCS,[24] which require doing toxicity testing without adjusting the effluent pH or adding buffers.

The brief exposure (15 min) of sperm and eggs of the green sea urchin to EGCS effluent at the lowest test concentration, i.e., 0.0001% (∑16 PAH = 16.19 pg L^{-1}), reduced fertilization success by 4%. Fertilization success in sea urchins has been proven sensitive to several components also found to be present in EGCS effluents. Krause et al.[55] observed a 10−20% reduction in fertilization success in the purple sea urchin (*Strongylocentrotus purpuratus*) when exposed to 0.0001% of produced water collected at the outlet of a petroleum oil processing site. The authors attributed the effects to the sensitivity of gametes, primarily spermatozoa, to petroleum compounds.[55] Warnau et al.[56] observed reduced fertilization success after exposing spermatozoa from *Paracentrotus lividus* to Cu, Cd, and Hg. Metal toxicity can be mediated by various modes of action where one of them is "ionic mimicry" leading to a competition between similar metal ions for binding sites on the surface of cell membranes.[22,57] Zn has been observed to disrupt cellular Ca^{2+} homeostasis through competition for ion channels.[22,58] In our study, it is not possible to attribute the effects to eggs or sperm since the gametes were exposed together. Both metals and PACs, observed to impair fertilization in other studies, are present in EGCS effluents, potentially acting in concert.

With increasing concentrations of the effluent, an increasing number of larvae developed abnormally, with malformations manifested as failure to complete gastrulation, as uneven arm length, absence of arms, unopened mouth, or deformed body shape (Figures 3 and SM S2). Abnormal larvae are less likely to reach a stage where they develop and live as normal larvae even after the pollution is gone, since their swimming and food-capturing ability is hampered by uneven or/and short arm length (Figures 3 and SM S2). Most larvae from the 5 and 10% EGCS effluent exposures did not have a developed gastrointestinal system at the pluteus stage (Figures 3 and SM S2), which also prevents them from feeding. Other studies have shown EGCS effluent exposure to hamper larval development in the two copepod species *Acartia tonsa* and *Calanus helgolandicus*.[20,23] Picone et al.[23] observed a delayed larval development in *A. tonsa* copepodite-I stage (C−I) at concentrations lower than 0.1% of OL-EGCS effluent (∑PAH 16 = 15.394 ng L^{-1}). Thor et al.[20] investigated the effects of EGCS effluents from two CL and one OL systems on the mortality and development of larval stages

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Environ. Sci. Technol. 2026, 60, 5231−5241

(copepodites, C) of the ubiquitous pelagic copepod *C. helgolandicus*. The authors observed unsuccessful molting from copepodite stages CIII to CIV already at the lowest CL-EGCS effluent and next lowest OL-EGCS effluent concentrations tested, i.e., at 0.1% ($\sum$PAH 16 = 16.085 ng L^{-1}) and 5% ($\sum$PAH 16 = 550.9 ng L^{-1}), respectively. Han et al.[59] explained reduced growth and development in the copepod *Tigriopus japonicus*, when exposed to the water-accommodated fraction (WAF) of Iranian crude oil, by the onset of energy-demanding processes like antioxidant enzyme activity (glutathione-S-transferase, glutathione reductase, and catalase) and cytochrome P450 gene expression. Similarly, Krause et al.[55] reported significant effects of petroleum oil-contaminated water at 0.0001% concentration on the development of *S. pupuratus* from the zygote to the pluteus stage at 4 dpf. In our study, larvae that managed to develop normally had a lower somatic growth rate, with EC_{10} at 0.88% and EC_{50} at 4.62%.

From the measured reduction in larval growth rate, it is possible to calculate the extra mortality caused by increasing EGCS effluent exposure. When larvae grow and develop more slowly, they spend more time in open water, subjected to predation before settling. Lamare and Barker[60] estimated the mortality rate in the New Zealand sea urchin (*Evechinus chloroticus*) in the field to be between 0.085 and 0.164 d^{-1}, mainly resulting from predation. Based on a 30-day developmental period before settling, sea urchin larvae would thus experience between 92 and 99% mortality.[61] The calculated effect of EGCS effluent exposure on mortality rate was 0.046 d^{-1} or 4.6% per day at an effluent concentration of 0.0001%, with a rapid increase between 0.01% and 0.1% to 0.38 d^{-1} (38% per day) at an effluent concentration of 0.1% (SM Table S4). The extra mortality rate was 0.87 d^{-1}, or 87% per day, at 2% EGCS effluent exposure. Thus, with a naturally high mortality rate, additional stress from EGCS effluent exposure would risk leading to extinction of the population already at very low concentrations when the presence of EGCS effluent and sea urchin reproduction overlaps in time and space. Modeling efforts by Aghito et al.[62] show that there is reason for concern in specific areas along the European coasts already today. Future predictions show increasing EGCS effluent levels in all European waters if restrictions are not implemented. Hampered growth also translates into a reduced larval biomass, which in turn may lead to food shortage for predators.[63] This is particularly important since the windows for growth and biological production are narrow in temperate coastal areas, and dependent on the season.[63] EGCS effluents have also been shown to affect species abundances and community composition in experimentally exposed plankton communities.[31,64,65] Such changes have the potential to affect higher trophic level species like cod, which has preferences for specific zooplankton species for food.[66,67] As a species, *S. droebachiensis* also plays a pivotal role in structuring subtidal ecosystems along coasts of both arctic and temperate waters and has been found in habitats from the shallow intertidal to 300 m depth.[68] Consequently, biological changes caused by EGCS effluent pollution may be complex, leading to shifts in food web interactions and trophic structures, which also may affect commercially relevant species.

Natural filtered seawater (0.5 μm) was used in all experiments of this study. Plankton larger than 0.5 μm were thus excluded from the exposure water, while the most abundant marine bacteria and archaea, being very small (normally ca. 0.3 μm in diameter and some nonspherical with the largest dimension <0.6 μm), would still be present. Using filtered seawater ensures constant exposure conditions and reduces the effects of confounding factors, which may otherwise obscure data interpretation, while at the same time retaining natural micronutrients, colloids, and most of the picoplankton community.[69,70]

Specific microbial communities may play an important role in the biodegradation of PAHs in natural systems.[71−73] However, this natural attenuation process may be limited under environmental conditions, where EGCS effluents are continuously discharged, leading to sustained exposure outpacing microbial degradation capacity. *S. droebachiensis* also spawns in early spring, where water temperatures are around +5°C and the temperature-dependent microbial activities are naturally very low.

This study included a limited subset of sea urchins collected in the field. While these individuals may not represent the complete genetic diversity of the population, samples from natural populations, as compared to laboratory-reared ones, will always provide a higher environmental relevance to the investigation. This study used effluent from one ship equipped with an OL-EGCS. The increasing body of knowledge regarding different types of EGCS effluents indicates that the compositions are fairly similar between ships but vary significantly in total concentration. Fuel type and engine load are also important factors affecting the effluent characteristics.[2,19,74] It is therefore fair to highlight the general importance of studies like the present, addressing the effects of single effluents. Thor et al.[20] measured effects of three different EGCS effluents on the marine copepod *Calanus helgolandicus*, showing that both OL- and CL systems produce effluents of comparable toxicity but with different magnitudes. This suggests that the harmful effects observed in our study may not be limited to a specific EGCS configuration but rather reflect a fundamental toxicity linked to EGCS effluents. Thus, although operational system differences exist, the potential ecological risks are likely to be inherent to EGCS.

In conclusion, we observed that EGCS effluent from an OL system had severe effects on early life stages of the sea urchin *S. droebachiensis*. Significantly reduced fertilization success was already observed in the sea urchin eggs at the lowest EGCS effluent concentration tested (0.0001%) and was primarily attributed to contaminants rather than to changes in alkalinity or pH. At concentrations below 1% of EGCS effluent, the buffering capacity of seawater is high, and the acidifying effects of the effluent are negligible. Stronger negative effects were observed at higher effluent concentrations, with significant arrested and abnormal development of larvae at 5% and 10% EGCS effluent, respectively. Exposure to EGCS effluent hampered the somatic growth of the larvae and thus delayed the time to reach the settling stage. This delay increases the risk of predation mortality, adding to the already high natural larval predation mortality (92−99%) and thus risking lowering the recruitment potential of natural sea urchin populations. The EGCS effluent induced mortality and hampered larval growth, which, in addition to the natural mortality, can harm whole populations. In fact, sea urchin populations started to decline in many coastal areas of the world around the current millennium,[75,76] in some instances coinciding with an increase in ship traffic and EGCS use.[77,78] Our results raise serious concerns regarding the impacts of EGCS effluents on marine life. By allowing the use of EGCSs and a sustained use of high-sulfur HFO, the IMO also allows for an increased impact of fuel-related pollution from shipping on marine ecosystems. This occurs at a time when reductions in

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Environ. Sci. Technol. 2026, 60, 5231−5241

marine pollution are urgently needed and shipping is bound to take on a sustainable path.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.5c02483.

Raw data on fertilization tests, developmental tests, growth to mortality, larval measurement, and effluent analysis (XLSX)

Experimental methods: serial dilution of EGCS effluent, larval anatomy, and carbonate chemistry of exposure water; results: fertilization success, photographs of abnormal larvae, and larval skeleton length (plots and allometries); discussion: calculated settling time and extra mortality of larvae in the wild (PDF)

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Notes

The authors declare no competing financial interest.

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