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Towards automatization in morphometric analysis of sea urchin embryos for ecotoxicological applications and near-future scenarios simulation

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ABSTRACT

Ecotoxicological bioassays are widely recognized as excellent tools for detecting the bioavailability and toxicity of many environmental pollutants. Specifically, embryo bioassays with invertebrates are among the most sensitive approaches used in the ecological quality assessment of the marine environment. However, these tests are time-consuming and expert judgment dependent, which potentially affects the results based on the ability of the operator and the number of counted embryos. These limitations can be overcome by developing a morphometric analysis based on image acquisition. Herein, a completely automated acquisition system of images for a fast and effective discriminant morphometric analysis is faced by using digital pictures of embryos of the Mediterranean Sea urchin *Paracentrotus lividus*. Linear Discriminant Analysis (LDA) was applied to find the best combination of automatically acquired morphometric parameters capable of discriminating among different morphological phenotypes by following the expert judgment. The automatized method shows a performance of 82% in detecting normal embryos vs a performance of manual observation of 79%. Our findings highlight the parameter area/perimeter ratio as the most critical descriptor to discriminate among different morphological phenotypes based on a predetermined classification covering six morphotypes. The method was validated on embryos exposed to elutriates from contaminated marine sediments. The high proficiency in classifying the embryos reveals the suitability of this method for the future assessment of marine pollution and complex scenario simulations.

1. Introduction

Anthropogenic activities impact the oceans, producing large amounts of pollutants with serious implications for marine ecosystems and human health. Alone, chemical investigation does not provide complete information on the biological risk of the new substances released in the environment on biological species and ecosystems (Schuijt et al., 2021). For this reason, nowadays, the importance of assessing the biological effects of contaminants is widely recognized as the necessity of focusing on an integrated approach combining chemical and biological methods for evaluating the potential biological hazard of single or mixed contaminants or multiple stressors (Bonaventura et al. 2021; Gambardella et al. 2021). Adopting a standardized ecotoxicological battery of bioassays is gaining momentum to objectively evaluate marine sediments and seawater quality rapidly, sensitively, and cost-effectively (Saco-Alvarez et al. 2010). In the last years, a multidisciplinary weight of evidence (WOE) approach was applied with success in several studies for the risk assessment of pollutants in the field (e.g., polluted marine sediments, harbor areas, complex natural and anthropic impacts on the marine environment) (Piva et al., 2011; d'Errico et al. 2021; Cecchetto et al. 2023). The WOE considers chemical analyses and ecotoxicological bioassays as different lines of evidence (LOEs) to express with synthetic indices determining the hazard level quantitatively integrated using weighing or ranking (Linkov et al., 2009). A weighted

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elaboration permits a qualitative classification of marine sediments and the indication of different management options; the crucial role of ecotoxicological bioassays in this approach is already included in the Italian law on dredging and dumping of sediments (DM 173/2016). Ecotoxicological data are typically obtained from different species, strains, exposure times, and endpoints, including survival, reproduction, and growth (Picone et al. 2016). Among the bioassays, the embryo test in marine invertebrate models is considered the one that's most reliable and widely used, as the embryonic developmental phase and the subsequent larval metamorphosis are vulnerable phases of the marine invertebrate life that pollutants can easily alter (Rakaj et al 2021).

In these standard toxicity tests, embryos are conventionally classified as "normal" or "abnormal" based on the percentage of embryos developing differently to controls, not discerning the differences (e.g., specific malformations, blocks, and delays in the development). This means that all that is different from the control is considered abnormal. To overcome this limitation, new analytical indexes were applied to the sea urchin embryo test by integrating the frequency of abnormal embryos with the severity of such abnormalities for weighting the teratogenic effects of contaminants (Morroni et al., 2016; Corinaldesi et al. 2017; Bonaventura et al. 2021, Morroni et al. 2023). Additionally, methods of analysis based on selective morphometric criteria (e.g., skeleton malformation) were developed in the sea urchin (Carballeira et al. 2012) as in other model organisms (e.g., fish, ascidian) (Jarque et al. 2020; Henrik Hansen et al. 2021; Gazo et al. 2021).

In sea urchins, the morphometric analysis is made at the pluteus stage, observing the correct development of several parts of the pluteus body, including the length of the longest postoral arm (POL), the body width (BW), and the body length, or considering the postoral arms' gap (POG) and the stomach volume (Dorey et al. 2013; Bertucci and Bellas 2021). For example, the body length was selected by Fernandez and Beiras (2001) as a robust criterion for evaluating the sea urchin embryotoxicity test because it is more reliable than the standard approach based on the percentage of normal embryos only. Based on these encouraging results, Bertucci and Bellas (2021) developed a "Growth Calculator" for larval-specific evaluation (POL).

Although these analytic methods improve the accuracy of the evaluation, they are less rapid and more complex than traditional toxicological observation of normal vs. abnormal embryos. Moreover, these methods remain traditionally hindered by the operator's manual acquisition, not to mention the personal judgment in the interpretation, potentially affecting the results, for example, due to a low number of counted embryos or a variable level of confidence with the system of the operators. This potential variability could have serious practical implications, considering the importance of embryo bioassays in evaluating the quality of sediments using an integrated approach with subsequent management decisions.

These limitations can be overcome by developing an automatized morphometric analysis system, which has already been performed for fish and sea urchin toxicity testing. For example, in adult rainbow trout *Oncorhynchus mykiss*, micro-computed tomography is applied to measure the volumes of the fish's different organs as a replacement for a manual morphometrics measurement and related calculation of the somatic indices (Brinkmann et al., 2016). Furthermore, a machine learning approach is developing to perform automated identification and indexing of all the endpoints for the fish embryo toxicity test evaluation using reach databases of digital images. In agreement, Dong et al. (2023) applied a deep learning-based morphometric analysis to quantitatively identify abnormal phenotypes and vital organ features of zebrafish embryos, finding an efficient hazard response and identification for chemicals and environmental pollutants. Alvarez-Mora et al. (2022) and Lopez-Herguedas et al. (2023) applied this approach to sea urchin embryo test in effect-directed analyses (EDA) strategy presenting a predictive system (SETApp) that collects embryos parameters and, estimates their malformation and size increase. However, to use a machine learning strategy as a robust tool to be introduced among the legislative requirements for ecological risk assessment it is necessary to implement and simplify the high throughput screening method.

With this background, the present study aims to: (i) develop an implemented automized acquisition procedure able to analyse images of a high number of embryos quickly and with high accuracy excluding artifacts due to operator load in embryos selection. (ii) set up an automatized analysis of developmental anomalies classifying embryos using six levels of severity (Morroni et al. 2023).

2. Materials and methods

2.1. Sediment sampling and elutriate preparation

Sediments were collected in May 2019 in Piombino harbor (North Tyrrhenian Sea, Italy). Sediment samples were stored at 4 °C and, within ten days from sampling, elutriates were prepared following USEPA (1991) guidelines and literature procedure (Broccoli et al. 2021). Specifically, sediment samples were mixed in a 1:4 (v/v) ratio of sediment to 0.45 μm filtered seawater (FSW) and placed on a rotary shaker table for 1 h, at a speed of 300 rpm, at room temperature. The FSW was collected in a long-term monitored reference site (Fortullino, Livorno, Italy) located far from sources of pollution from human activities. After mixing, the samples were centrifuged (Thermo Scientific SL 16R, Rodano, Italy) for 20 min at 3000 rpm (4 ∘C), and the aqueous fractions (elutriate samples) were collected and stored at −20 ∘C until the use to perform the sea urchin embryo bioassay.

2.2. Embryol bioassay and experimental set-up

Adults of *P. lividus* were collected at Santa Marinella in the central Tyrrhenian Sea, Italy (42°3′0″N, 11°49′9″E). After collection, the sea urchins were transported in separate containers to the Laboratory of Experimental Ecology and Aquaculture (University of Rome Tor Vergata) and acclimatized for one week in flowing seawater at a temperature of 15 °C ± 1, salinity 36 and natural photoperiod (12:12 h). Embryotoxicity tests were performed following Morroni et al. (2018). Twelve males and twelve females were induced to spawn by injecting 1 ml of 0.5 M KCl into the sea urchin body cavity through the peristomial membrane around the mouth. Eggs were collected by placing spawning females on 100 ml beakers of filtered seawater, which had been collected from the same site as the sea urchins. Females with immature eggs, not round or with debris, were discarded. Sperm was collected "dry" from the gonopore of male sea urchins and was kept on melting ice until use (<1h). After checking sperm motility and the success of fertilization (>95 %), 50 μl of sperms were diluted in 5 ml of FSW and added to 350 ml of egg suspension (1000 eggs/ml), sperm/egg ratio 50:1 (Morroni et al. 2018). After 20 min following the fertilization, incubations began with test solutions in 10 ml sterile capped polystyrene six-well micro-plates (1 ml per well, corresponding to a final density of about 100 embryos/ml), at a temperature of 20 °C in a thermostatic chamber for 48 h. In the control condition, embryos were exposed to FSW only. Tests were accepted if the percentage of control embryos (negative control) was 70 % (Morroni et al. 2016). Copper (Cu) was used as a reference toxicant in positive control, diluting 1 g/L stock solution (Sigma Aldrich srl, Milan, Italy) in bi-distilled water (BDW), obtaining a concentration of 10 mg/L (Morroni et al. 2023). Test solutions were obtained through increasing dilutions in 10 mL of FSW (0.01, 0.02, 0.03, 0.04, 0.05, 0.06 mg/L), and tests were accepted if they fell within the laboratory acceptability ranges (Morroni et al. 2018; Rakaj et al. 2021). Elutriate samples from Piombino harbour (North Tyrrhenian Sea, Italy) sediments were prepared, using 3 dilutions in FSW (90 %, 50 %, 25 %), with 6 replicates per dilution, estimate dose–response curves (Rakaj et al. 2021).

2.3. Sample preparation and image acquisition

Once reaching the setting endpoint (4-arm pluteus stage), the samples were fixed using Lugol's iodine solution to optimize the photo acquisition and obtain well-contrasted embryos images. After fixation and embryos deposition on the bottom of each multiwell, 3/4 of the supernatant liquid was removed by aspiration to avoid image blurring due to the Lugol fixative. Fixed samples were then photographed, capturing more than 50 embryos per picture, by stereoscope and inverted microscope (Axio Zoom V16 and Zeiss Axio Vert A1) equipped with digital cameras (Zeiss Axiocam 105). Image acquisition was performed employing standardized settings with 10x magnification. A white background was used setting the AUTO white image balance and AUTO set exposure. In the display tab of color channels, the AUTO Min/Max option allowed us to keep the whole spectrum in black and white, with black (contrast) as the smallest intensity, white (brightness) as the largest, and the gamma value of 0.45. These settings ensured high image quality across all samples. To analyse at least 300 embryos in each single well, three pictures of different well-bottom areas (with more than 100 larvae) were collected. The images were acquired from all the replicas of each experimental condition (1000 larvae analysed for each one), allowing to record of the effects derived by the tested stressors.

2.4. Automatized image analysis

Image analysis was performed using ImageJ software by following a sequence of steps to obtain automatically morphometrical descriptors from embryos. Images were firstly converted to binary format (Fig. 1A-B) to facilitate data acquisition and any gaps or holes inside the embryo structures were filled in black (Fig. 1C). All objects cut by the image edges were automatically removed and excluded from the analysis and objects smaller than 70 % of the mean egg area were removed to eliminate noise and small particles. All the objects with an area larger than 130 % of the mean endpoint embryos were also automatically removed from the analysis to exclude the overlapped embryos (Fig. 1D). All data from the remaining objects (embryos) were finally acquired, including major axis, minor axis, area and perimeter (Fig. 1E). ImageJ software was used to automatically acquire these parameters from the pictures,

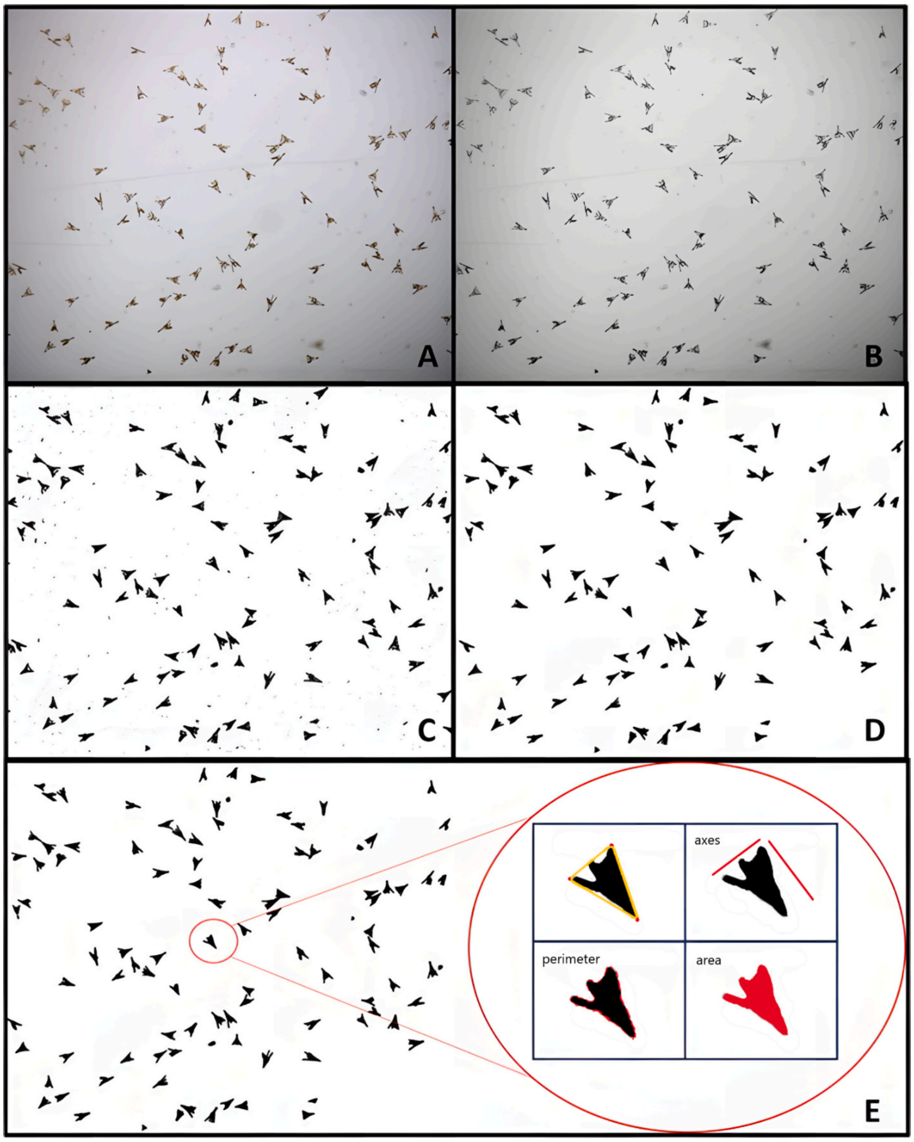

Fig. 1. Step of automatic image conversion for acquiring morphometrical parameters in *P. lividus* embryos. A: Starting image, B: black and white, C: Image with structures of the closed perimeter (inside the embryo) filled in black: D: Image with the left embryos after elimination of objects smaller than 70% of the mean egg area and larger than 130 % of the mean endpoint embryos, E: Morphological parameter acquisition. This representation derived from a sub-area (a quarter) of the original image.

demonstrating its capabilities in handling complex image processing tasks.

2.5. Standard vs automatized embryo analysis

Each embryo in each image was classified by expert judgment according to six levels of severity following the toxicity scale of ITI reported by Morroni et al (2023). At the same time for each embryo, after classification, the major axis, minor axis, area, perimeter, and area/perimeter ratio were acquired automatically. The first step involved selecting the larvae using a pointer that moved pixel by pixel. When the pointer detected an embryo-larva, its morphometric parameters were extracted. Firstly, the perimeter and area of the embryo-larva were measured. Next, the embryo-larva underwent a roto-translation process to optimally align it within an isosceles triangular framework. The major axis was then measured as the height of the triangle, while the minor axis was recorded as its base. This procedure allowed for the acquisition of all the morphometric parameters used in the study as number of pixels (Supplementary Materials_Table1). Considering that ITI classification assigns a score ranging between 0 (none) and 5 (highest severity) to randomly chosen embryos from each condition, a score of 0 was assigned for the pluteus stage, 1 to the delayed stages corresponding to early pluteus, 2 for malformed pluteus, 3 for early malformed pluteus, 4 for prisms and 5 for blastula, gastrula and morula (see Fig. 2 for the complete embryo development up to pluteus stage).

Following this step-up, morphometric descriptors were analysed and classified with expert judgment to define the best combination of descriptors, synthesizing the progressive variation of ITI in the dose–response curve. With this aim, Linear Discriminant Analysis (LDA) was carried out using the LDA function of the MASS package in R. This multivariate data analysis calculates the best linear combination of descriptors to separate the six ITI classification groups of embryos objectively, assigning each point to the group that gives minimal Mahalanobis distance to the group mean. The Mahalanobis distance was calculated from the pooled within-group covariance matrix giving a linear discriminant classifier. Five morphometric parameters (major axes, minor axes, perimeter, area, and area/perimeter ratio) were tested as discrimination factors for the end-point embryos classification in 6 groups: 0 (well-developed pluteus); 1 (malformed pluteus); 2 (early pluteus); 3 (early malformed pluteus); 4 (prism); 5 (eggs, blastula, and gastrula, presenting round structure) (Fig. 3). At this step, the quality of the allocation of unknown objects to the groups was assessed (supervised learning) and classified as positive and negative.

The automatized embryo analysis was further validated using dose–response curves generated from case-study elutriates sourced from Piombino harbour. The *P. lividus* bioassays were conducted with four different elutriates at varying concentrations 0 %, 25 %, 50 %, 100 % (elutriate:seawater, v/v). At the end of the experiment, six images from each condition were analysed in parallel through expert judgment and automatic reading. This resulted in a total of 108 images and approximately 120 00 larvae being used to construct the dose–response curves for each methodology. This comparison enabled to investigate the accuracy and reliability of the automatic methods compared to expert readings.

3. Results

3.1. Automatic model outcomes

The new protocol involved fixing samples using Lugol's iodine solution instead of formalin once they reached the setting endpoint (4-arm pluteus stage). This change allowed for optimizing photo acquisition and obtaining well-contrasted embryos images. Lugol's solution makes embryos more translucent and easier to analyse with image analysis software.

Regarding the time required for analysis using expert judgment, it

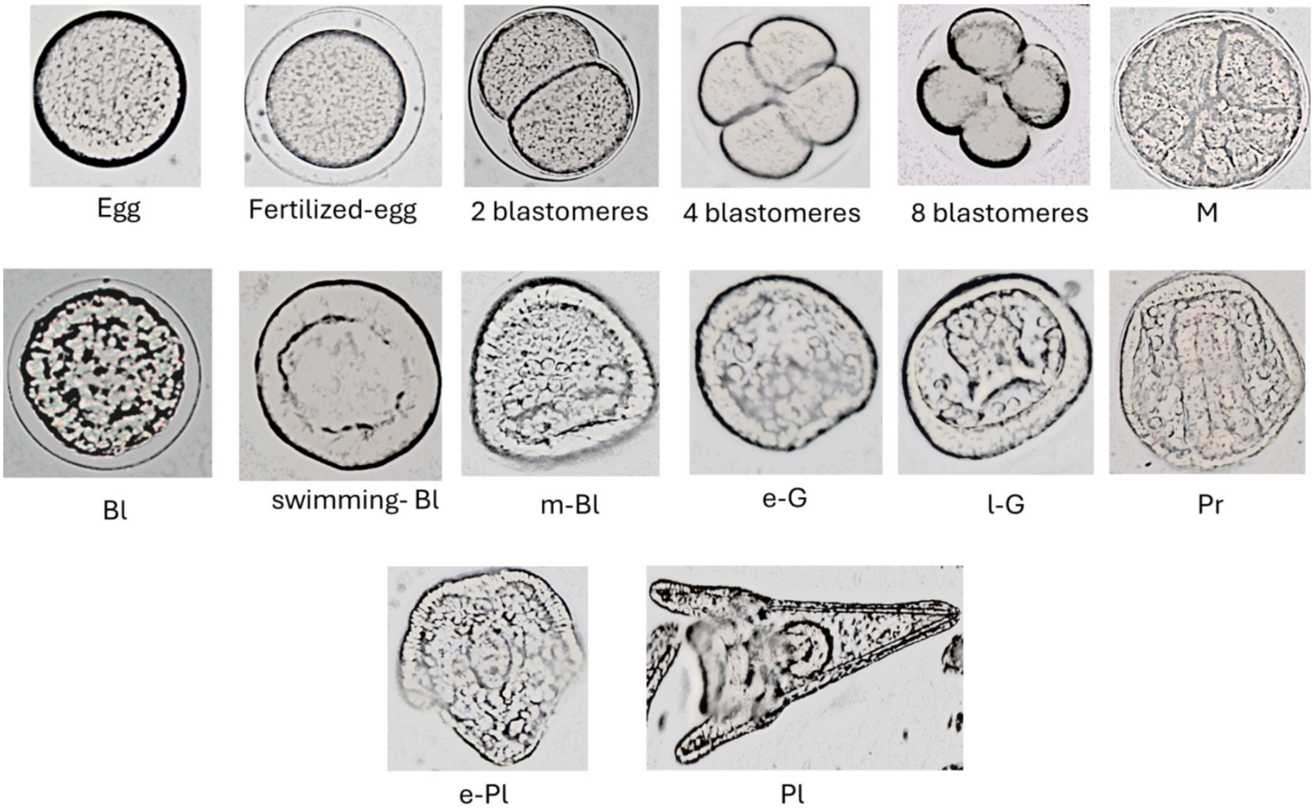

Fig. 2. Sea urchin *Paracentrotus lividus* embryo development. M: Morula; Bl: Blastula; m-Bl: mid Blastula; e-G; early Gastrula; l-G: late Gastrula; Pr: Prism; e-Pl early Pluteo; m-Pl mid Pluteo; Pl:Pluteo.

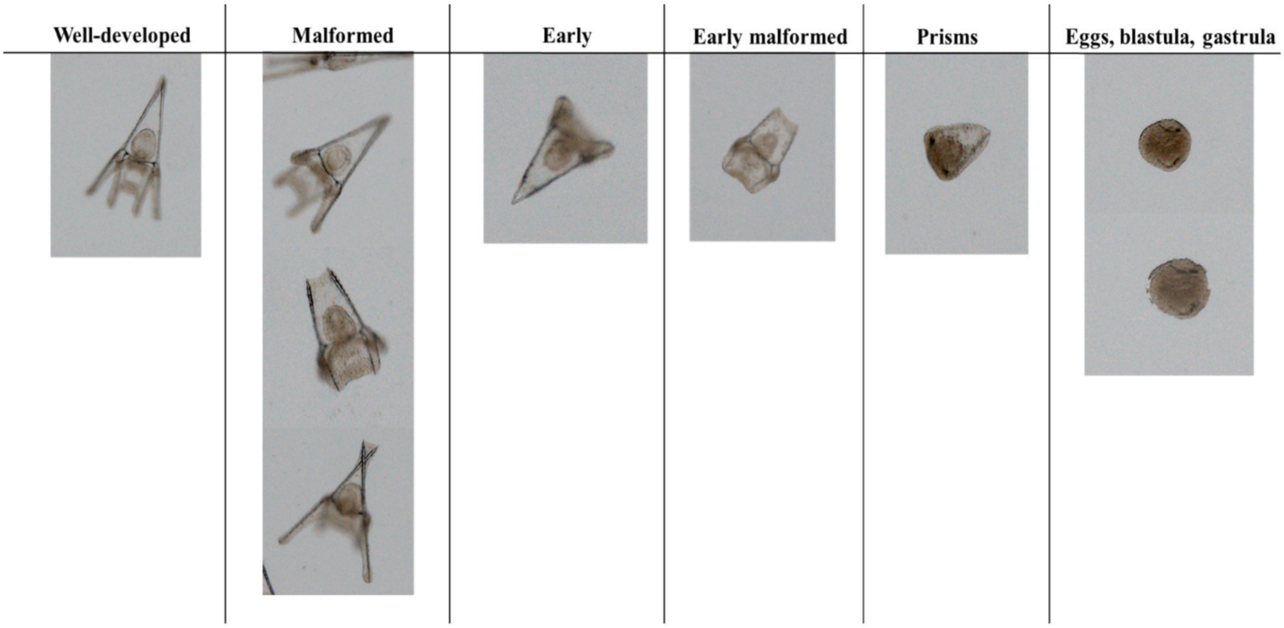

Fig. 3. Morphotypes and developmental anomalies in *P. lividus* embryos in each classification group. The example photos shown are the images used for automatic acquisition.

was found that approximately three hours were needed to classify 1000 larvae (the number classified for each experimental condition) following the ITI protocol (Morroni et al., 2023). In contrast, the same number of larvae were processed by the automated approach in less than one minute, demonstrating a significantly time-saving alternative compared to expert judgment.

The results of the Linear Discriminant Analysis (LDA) are shown in Table 1 and Table 2. The outcomes of the training activity for identifying well-developed pluteus are presented in Table 1. The performance of the automatized methods increased up to 77 % if applied to detect the standard endpoint of well-developed pluteus (60 well-identified plutei on 78 total embryos), with 87 % in the detection of abnormal embryos (225 well-classified embryos on 257 total embryos), demonstrating an average performance of 82 % (Table 1). Analysing the same endpoint, the manual observations performed by the operator showed a performance of 65 % in recognizing well-developed pluteus (60 on 92 embryos) and of 93 % in the identification of abnormal embryos (225 on 243 embryos), with an average performance of 79 % (Table 1). Additionally, the test set exhibited the same rate for well-identified pluteus, estimated at 77 % based on the information provided in the confusion matrices. The corresponding values of Intraclass Correlation Coefficient (CCI) and K-means (K), respectively 0.85 and 0.61, were comparable to those of the training set. The model's capacity to distinguish between the two classes is evident in the graph presented in Fig. 4, where the two curves represent the density distribution of malformed and well-developed individuals in the provided data. The minimal overlap between the two curves indicates the model's clear discriminatory ability.

Table 1
Evaluation of the performance of the automated method and operator standard classification method of morphotypes based on the results of multiple jackknife runs. Confusion matrix of the binary model, with training set above and test set below. Data are expressed as the number of identified embryos for each morphometric group. Standard measurements with the operator are reported in the columns, and automatic method results are reported in the rows. B.

Training	Standard vs Automatic	1	2	Total
CCI 0.86	**1**	137	41	**178**
K 0.66	**2**	48	446	**494**
	Total	**185**	**487**	**672**
Test	**Standard vs Automatic**	1	2	**Total**
CCI 0.85	**1**	60	18	**78**
K 0.61	**2**	32	225	**257**
	Total	**92**	**243**	**335**

Table 2
Confusion matrices are used to evaluate five classes of morphotypes compared with ITI classification, with training set above and test set below. In this table, morphotype classes are represented as follows: 0 – well-developed pluteus, 1 – early pluteus, 2 – malformed pluteus, 3 – early malformed pluteus, 4 – prism, 5 – eggs, blastula, and gastrula.

Training	Standard vs Automatic	0	1	2	3	4	5	Total
	0	142	4	44	0	0	0	**190**
	1	6	60	23	28	0	0	**117**
CCI 0.63	**2**	37	16	66	2	0	0	**121**
K 0.69	**3**	0	21	12	82	5	1	**121**
	4	0	1	0	10	36	18	**65**
	5	0	0	0	1	16	41	**58**
	Total	**185**	**102**	**145**	**123**	**57**	**60**	**672**
Test	**Standard vs Automatic**	**0**	**1**	**2**	**3**	**4**	**5**	**Total**
	0	69	0	20	0	0	0	**89**
	1	0	30	12	9	0	0	**51**
CCI 0.63	**2**	23	10	35	0	0	0	**68**
K 0.68	**3**	0	11	5	42	7	0	**65**
	4	0	0	0	8	15	11	**35**
	5	0	0	1	2	6	18	**27**
	Total	**92**	**51**	**73**	**61**	**28**	**30**	**335**

In terms of identifying five classes of morphotypes, the training set successfully identified, against expert judgment, 75 % of four-arm pluteus, 51 % of malformed pluteus, 55 % of early pluteus, 68 % of early malformed pluteus, 55 % of prisms, and 71 % of eggs, blastula, and gastrula. The confusion matrix results of the test set for identifying different morphotypes of pluteus are presented in Table 2. The automated method achieved identification comparable with the training ones, with frequencies of 78 % for well-developed pluteus, 59 % for malformed pluteus, 51 % for early pluteus, 65 % for early malformed pluteus, 43 % for prisms, and 70 % for eggs, blastula and gastrula. Overall, this indicates a good capacity for discrimination among classes. The values of CCI and K between the training and test sets are comparable, with values around 0.63 for CCI and 0.68 for K. The scatter plot in

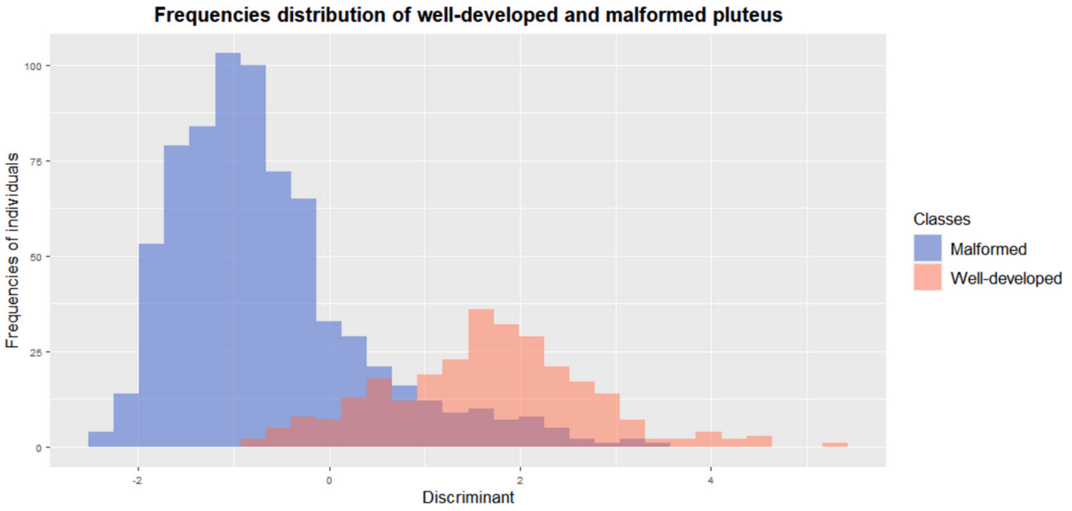

Fig. 4. Density diagram representing the distribution of two classes: malformed plutei (blue) and well-developed plutei (red). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

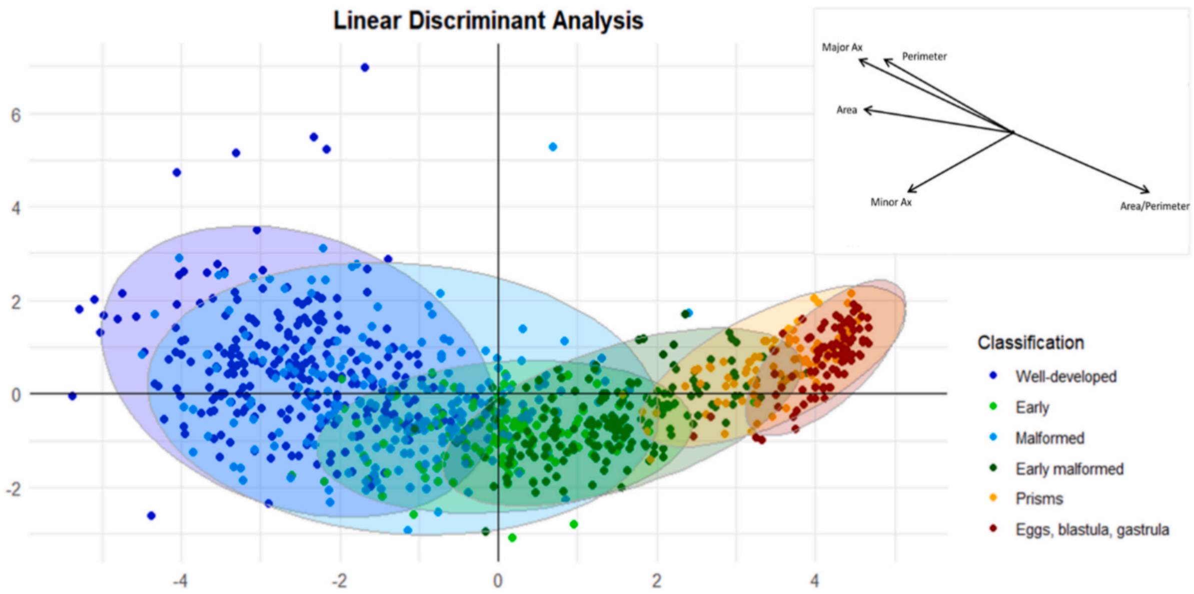

Fig. 5. LDA results in a scatter plot of five classes of morphotypes using perimeter, area/perimeter ratio, area, minor axes, and major axes as discriminant parameters.

Fig. 5 generated through Elliptic Fourier analysis reveals five distinct classes of morphotypes, demonstrating how the model accurately separates the different classes. The explained variance for axis 1 is 90.97 % and 6.37 % for axis 2. The classification is influenced in order by perimeter, area/ perimeter ratio, area, minor axes and major axes; notably, for both axes, the parameters that contribute most are perimeter and area/perimeter ratio.

3.2. Validation of classification model

A dose–response curve was generated using case-study elutriates from the Piombino harbor to evaluate the model's ability. Both expert and automated classification were performed on data, and the results remarkably overlapped (Fig. 6). The assessment of embryotoxicity via the ITI method, which differentiates between six classes of severity reveals a more pronounced morbidity scenario compared to the curve based solely on malformed versus well-developed embryos. This emphasizes the ITI method's sensitivity in discerning varying degrees of severity and providing a clearer outcome. In this context, automated methods produce a more sensitive and responsive curve, showing its potential as a highly perceptive tool.

4. Discussion

The sea urchin is a common model species extensively used in developmental biology and ecotoxicology studies due to its simple manipulation and well-recognized embryos. The echinopluteus is characterized by bilateral symmetry with specific shapes, transparency, and easy availability in large amounts. These characteristics make it an ideal model for biotechnological applications. In the present study, a protocol based on sample manipulation and image acquisition was developed to automatize the morphometric embryonic analysis. Lugol solution, the fixative chosen, allowed the replacement of other substances without involving dangerous inhalations (e.g. formalin), preserving the intact anatomy of the embryo and colouring it for image acquisition by a stereo and inverted microscope. This approach allows contrasting of the

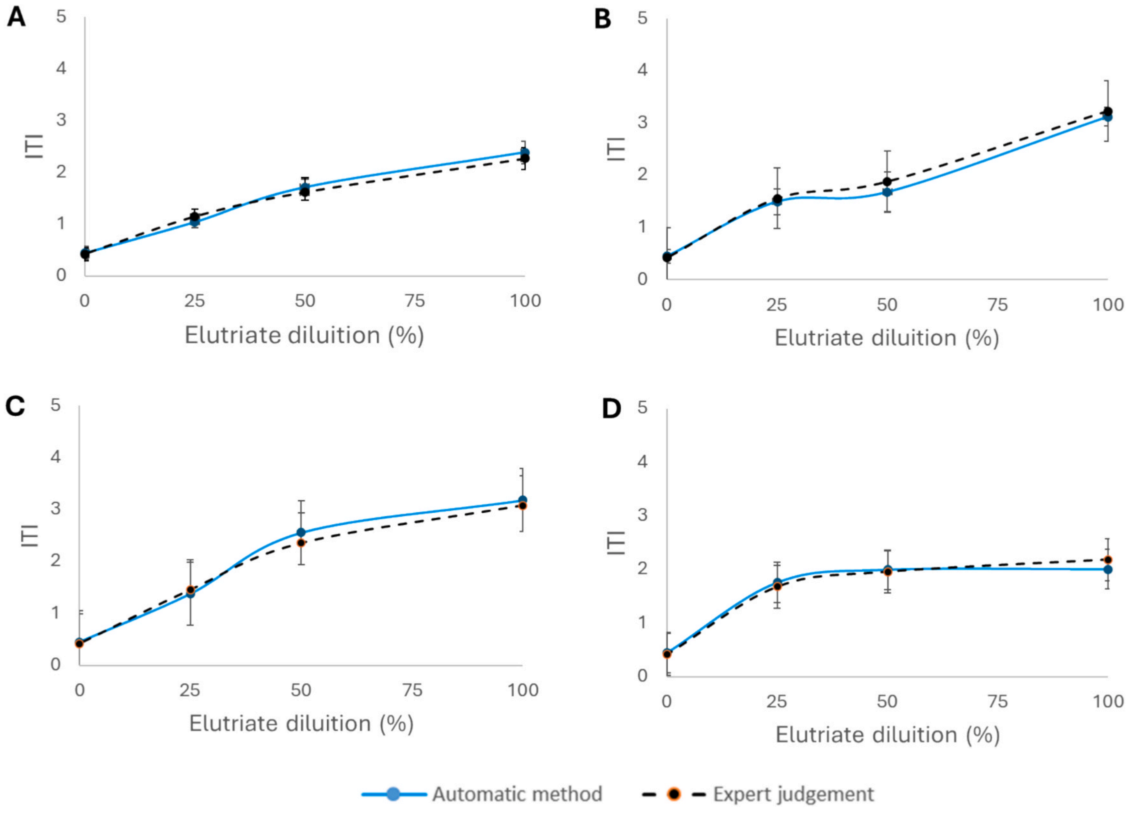

Fig. 6. Dose-response curves of four different elutriate samples from the Piombino harbour (A, B, C, D). The black dashed line represents the classification by expert judgement, while the blue line represents the classification by the automatic model. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

images complying with the standards required by a binary acquisition for image analysis. These improvements necessary for the automatized image acquisition development proposed in this study can be replicated and implemented in any laboratory without investing in *ad-hoc* technological equipment.

Image analysis performed using a sequence of steps in the ImageJ software facilitates image acquisition and data extraction for automatic classification. The steps include i) the image conversion into binary form; ii) the remotion of all background noise by eliminating objects smaller than eggs; iii) the remotion of objects with an area larger than the mean endpoint embryo to eliminate cases of overlapped embryos. These features allow the automatic removal of misleading particles and overlapped embryos, focusing the analysis on the remaining targets. These developments allow for significant advancement by overcoming the operator check of the single embryo images required by previous approaches (Alvarez-Mora et al. 2022), providing fully automated image acquisition without the involvement of an operator, with consequent reduction in time and increased possibility of processing a high number of embryos. The automated tool could be applied to a rapid and massive analysis of images (less than1 min per tested condition, ~1000 larvae), eliminating the need for time-consuming, one-by-one operator assessments (around 3 h,~1000 larvae).

Hence, the application of this new methodology for calculating morphological parameters (major axis, minor axis, area, perimeter, and area/perimeter ratio), commonly used in embryo-larval bioassays, has provided a robust, time-efficient, and reproducible approach for analyzing larval morphology. Given the large sample sizes typically involved in ecotoxicological studies, this method serves as an efficient and intuitive tool for processing and analyzing morphological data without requiring expert input or complex software. This is particularly advantageous for large-scale studies and high-throughput analyses, where efficiency is essential. Additionally, the simplicity and accessibility of these metrics enhance their practical value in decision-making and communication with key stakeholders, including environmental regulators and conservationists.

However, while this methodology provides a solid baseline for morphotype classification, future advancements will require enhanced computational approaches, such as Elliptic Fourier Analysis (EFA), to further improve resolution and discrimination accuracy in the automatic classification of larvae into the appropriate ITI categories.

Linear Discriminant Analysis (LDA) used in this paper for embryo classification is a factorial method applied to known groups of objects (in this case, embryos classified according to expert judgment) to find the best combination of morphometric descriptors to discriminate the groups. Furthermore, this method can automatically allocate new objects (embryo) to the defined groups using the same descriptors. The results demonstrated a strong performance of the automated method, with a CCI of 0.85 for the binary test, indicating an excellent ability of the automatic classification to distinguish between malformed and well-developed pluteus. The CCI value (0.63) decreases when classifying into six classes of phenotypes. However, it is an optimal choice considering the gain in resolution, and, despite this lower value, the quality of discrimination remains excellent for the test set. Frequencies of correct identification of the morphotypes were assessed for all classes and ranged from equal to or above 50 %. Overall, the sensitivity of the automatic methods stands at 76.9 % for binary classification and a mean value of 56.4 % for classification into six morphotypes. Among the stressors considered in this study, parameters of embryonic perimeter and area/perimeter ratio resulted as the most important descriptors to differentiate between morphotypes, followed by the area, minor axes, and major axes. In line with our findings, previous research has also recognized the efficacy of automated methods in biological classification. For instance, Tan et al. (2021) utilized LDA analysis to assess different species of cephalopods using various metrics from upper, lower, back, and left lateral views. Similarly, Brito de Jesus et al. (2023) employed different analytical approaches to develop automated methods. Specifically, they utilized random forest analysis to identify up to 100 species of nematodes. As with these previous findings, our results contribute to the exploration of automated techniques in ecotoxicology. The present study demonstrates that this innovative method allows a high-performance classification of embryos, standardizing data analysis acquisition without an operator in embryo bioassay. Initially, expert judgment was used to validate and establish a robust automated method for morphometric analysis, which subsequently became operator-free. This groundbreaking method shows superior capabilities concerning the existing techniques to build models to differentiate morphological features during sea urchin early embryogenesis and embryo development, classifying embryos in six different classes, achieving a more precise interpretation of teratogenic effects compared to the standard endpoints adopted in the current literature (Carballeira et al. 2012; Lopez-Herguedas et al. 2023).

Automatized classification tools, therefore, help to overcome important constraints in ecotoxicological bioassay application because they are rapid, precise, and accurate, enabling the analysis of higher numbers of embryos compared to a traditional toxicological testing strategy based on the observation by an operator (Dong et al. 2023). However, further investigations are needed to expand our understanding of the specific weight of each morphometric descriptor in defining the six ITI categories and to explore potential improvements to this automatic approach by integrating other proposals that used other computational tools (Collins, 2022; Rendell-Bhatti, 2021; Brito de Jesus et al. 2023; DeMiguel-Jiménez L et al. 2023). For instance, machine learning approaches, such as Random Forest and Inference Trees, can aid in identifying the most effective descriptors for defining malformation categories. Additionally, they can evaluate the contribution of different parameters to the classification of morphological abnormalities (whether as correlated or independent discrimination factors) and determine the numerical values of descriptors that characterize each morphotype and abnormality.

In a future perspective, automated data acquisition may also enable the assessment of effects that are currently not detectable due to a low incidence and the need to analyse a large number of embryos. Similar slight effects on embryos might be relevant in future scenario simulations such as those reflecting ocean acidification, temperature/salinity increase, or emerging pollutants (Gambardella et al. 2021). Correlation networks between specific embryo phenotypes and toxicity could be important to evaluate the hazard of complex media, such as mixtures of chemicals, or their interaction with physical factors.

Finally, our findings also lead to important consequences from an applicative perspective. Specifically, bioassays are essential components for evaluating sediment quality class in harbour areas, an issue of critical importance in these environments with strategic economic and environmental implications (Morroni et al. 2020). European Directives and various international agencies (e.g., OSPAR, HELCOM, MEDPOL, ICES) encourage multidisciplinary studies based on the integration of chemical measurements and bioassays, representing an added value to monitoring and management (Benedetti et al. 2012). For example in Italy, the decree on the management of dredged sediments (DM 173/2016), based on a WOE approach, considers chemical analyses and ecotoxicological bioassays as different LOEs through a quantitative integration. As a result of weighted elaboration and quality classification of marine sediments, different management options can be adopted according to dumping legislation (d'Errico et al. 2021). In this context, the present study proposes a protocol for a machine learning application in *P. lividus* embryo bioassay, improving its accuracy, facilitating data acquisition by many entities, such as environmental agencies, and thus boosting its practical use in the assessment of the environmental quality of harbour sediments. Additional studies would provide useful insights to further improve this tool toward a user-friendly specific software that could easily allow applying this protocol to images acquired by others, facilitating interlaboratory comparisons and further reinforcing the suitability of this method in ecological risk assessment.

5. Conclusions

In conclusion, we have developed a comprehensive automatized data acquisition from embryos and an operator-independent analysis of sea urchin developmental anomalies. A successful data acquisition protocol was established through the following steps: i) contrasting the embryo with a dye fixative, ii) automatic removal of the misleading particles and overlapping embryos by imaging software, iii) automatic classification of the severity of malformations in embryonic development by linear discrimination analysis (LDA). This innovative technique enabled a high-performance classification of embryos compared to traditional methods, standardizing data analysis acquisition without requiring expert judgment for embryo bioassays. In addition, we have validated the protocol with contaminated sediment from Piombino Harbour (Italy), thereby evidencing the method's effectiveness. Therefore, the present study demonstrates that this is a rapid, precise, accurate, and reproducible tool that can be adopted to assess marine pollution and near-future simulation scenarios.

CRediT authorship contribution statement

Arnold Rakaj: Writing – review & editing, Validation, Resources, Project administration, Methodology, Investigation, Formal analysis, Conceptualization. **Lorenzo Morroni:** Writing – original draft, Validation, Project administration, Methodology, Formal analysis. **Gaia Flori:** Writing – review & editing, Investigation. **Luca Grosso:** Writing – review & editing, Investigation. **Annalisa Pinsino:** Writing – review & editing, Validation. **Daniele Ventura:** Writing – review & editing. **Alessandra Fianchini:** Writing – review & editing, Investigation. **David Pellegrini:** Writing – review & editing. **Francesco Regoli:** Writing – review & editing, Validation. **Michele Scardi:** Writing – review & editing, Validation, Methodology, Investigation, Formal analysis, Conceptualization.

Declaration of competing interest

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

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecolind.2025.113533.

Data availability

Data will be made available on request.

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