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The spray technique: a new method for an efficient separation of fish eggs from plankton

A. T. G. W. ELTINK*[†]

INSTITUTE FOR MARINE RESOURCES AND ECOSYSTEM STUDIES (IMARES), PO BOX 68, 1970 AB IJMUIDEN, THE NETHERLANDS

[†]PRESENT ADDRESS: WEIDE 21, 1841GV STOMPETOREN, THE NETHERLANDS

*CORRESPONDING AUTHOR: guus.eltink@freeler.nl

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A fast automatic new method, the "spray technique", for the separation of fish eggs from plankton samples has been developed; each egg-sorting procedure requires less than 3 min (removal of zero up to hundreds of eggs). A targeted accuracy can be achieved by repeating the procedure. The spray method can be easily applied onboard research vessels and it appears to cope well with different plankton compositions. Tests suggest that the method is 25, 60 and 110 times faster than the traditional manual method when using samples with low, medium and high plankton content. Sorted egg fractions contain less contamination by plankton particles than with the manual method. This new method is much less prone to human error and can be standardised. However, as plankton samples vary, it is crucial to estimate regularly the accuracy of the sorting. The method should only be used with standard operating procedures and pre-determined accuracy targets. Accuracy can be estimated afterwards by sorting thoroughly; checking whether any eggs had been left.

INTRODUCTION

Ichthyoplankton surveys are used in a range of research areas in marine biology and fisheries science (Heath, 1992). All of them rely on the accurate determination of ichthyoplankton abundance in the samples. Within fisheries research both the daily egg production method (DEPM) (Parker, 1980; Lasker, 1985; Somarakis *et al.*, 2004) and the annual egg production method (AEPM) (Lockwood *et al.*, 1981; ICES, 2005, 2006) can be used to estimate biomass of spawning stocks. Some ichthyoplankton surveys used for egg production estimates generate large numbers of samples that need to be sorted for fish eggs and this often requires a large investment of human resources for manual sorting. The traditional method of sorting the eggs from a plankton sample used to be a tedious and very time-consuming job, because the fish eggs, present in mixed plankton samples, have to be recognised by eye and subsequently have to be removed manually. The quality of sorting is rarely tested and documented.

Automatic sorting methods for plankton have been developed earlier mainly for the purpose of sorting fish larvae from the rest of the plankton (Bowen *et al.*, 1972; Price *et al.*, 1977; Mamhot *et al.*, 1988). However, the information is scarce on egg-sorting methods and it is especially difficult to compare these sorting methods regarding efficiency and accuracy of egg sorting.

The new egg-sorting technique reported here uses a spray of aerated seawater for the removal of fish eggs from plankton samples. It is therefore called the "spray technique". It is based on the phenomenon that the tiny air bubbles attach to the parts of the plankton that have projections (pleopods, antennae, setae and other appendages), but do not attach to the smooth surface of the fish eggs. The aerated plankton floats to the surface and all smooth structures such as fish eggs sink to the bottom. However, although most of the eggs do indeed sink to the bottom, some become entangled in between

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the floating plankton. This fraction of entangled eggs probably depends on a number of factors. These include the volume of plankton, the buoyancy of the fish eggs, the species composition of the plankton, the duration of the 4% formaldehyde fixation, the specific density of the seawater used for spraying and the degree of aeration of the seawater.

The aim of this study has been to investigate and document the differences between this new spray technique and the traditional manual egg-sorting method in relation to the amount of plankton and the egg sorting of mackerel (*Scomber scombrus*) eggs. Therefore, all egg-sorting experiments were carried out with the same set of three plankton samples, which did not differ in species composition, but which only differed in plankton quantity. Consequently, differences in results of egg sorting from these plankton samples can only be attributed to differences in the amount of plankton and therefore enable a good comparison to be made of both egg-sorting methods. Both accuracy (bias) and precision of both egg-sorting methods can be calculated because all egg-sorting experiments have been carried out in triplicate.

With the manual method, it is very easy to pick out plankton at the same time as fish eggs, which then results in an egg sample contaminated with plankton. The spray method was also compared to manual sorting in terms of plankton contamination of the egg samples.

The bias and precision of the two methods and factors that impact on these are also described. The analysis of bias results in guidelines being constructed for the future use of the spray method and for the quality control of egg sorting.

METHOD

Equipment required for the spray technique

A water jet filter pump was used for the application of the spray technique, because it produced a spray of aerated seawater (Brand cat. no. 159600; www.brand.de). According to its technical description, it uses 190 L h^{-1} water (at 3.5 bar), whereas it sucks in air at a rate of 400 L h^{-1}. This implies that the aerated seawater spray from it can consist out of seawater mixed with about twice the volume of air. This water jet filter pump was mounted on a spray gun (Hozelock part no. 2667; www.hozelock.com), which has an easy opening and closing device (Fig. 1A). The salinity of the seawater used for the egg-sorting experiments was ~34.5. The egg-sorting device as shown in Fig. 1B is in fact a separating funnel, which consists of a transparent cylinder mounted on top of a transparent funnel including a tap on the bottom opening. A flexible silicone tube is mounted to the tap for the transportation of the fish eggs into a glass beaker. The device can store a volume of ~1.6 L. The inner diameter of the stem of the funnel is 25 mm. The inner diameter of both the tap and the silicone hose is 19 mm to allow larger particles in the plankton to pass through after the last sorting procedure when all plankton is transferred back into the sieve.

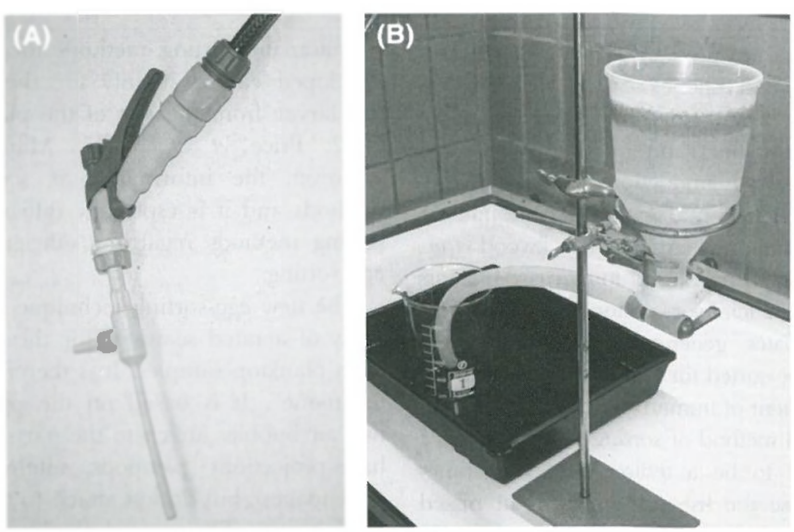

Fig. 1. (**A**) The spray of aerated seawater is produced by a water vacuum filter pump, which is connected to a spray gun. (**B**) The egg-sorting device for separating fish eggs from a plankton sample is a separating funnel. After spraying with aerated seawater, the plankton is floating, while the fish eggs sink to the bottom. The seawater with the fish eggs can be transferred into a 2 L glass beaker, which is placed in a black tray with water to enhance the visibility of the fish eggs (Fig. 2).

Preparation of the plankton samples prior to the comparative egg-sorting experiments

The plankton and eggs used for the egg-sorting experiments were obtained from 10 plankton samples collected during the mackerel egg survey in the western part of the central North Sea in June 2005. These samples contained high numbers of mackerel (*Scomber scombrus*) eggs, which have an egg diameter of ~1.2 mm. The wet weight of the plankton (predominantly crustaceans) in these samples ranged from ~10 to 65 g. Thousands of mackerel eggs had been removed from these samples and these were transferred into a jar with a 4% solution of buffered formaldehyde. This was then the stock of mackerel eggs, which was used for all egg-sorting experiments. This ensured that differences between experiments could not be attributed to differences in eggs, e.g. the buoyancy regarding the spray method and the transparency and translucency regarding the manual sorting method. The buoyancy (Coombs, 1981) and transparency are dependent on egg stage (Lockwood *et al.*, 1981) development. The egg-sorting experiments were carried out ~4–5 months after the eggs had been collected during the survey. The plankton of these 10 samples was well mixed in a glass beaker and then sieved. The mixing of the plankton was carried out beforehand to ensure that the composition of the plankton would be similar for all egg-sorting experiments. Three jars were filled with 10, 40 and 100 g wet weight of plankton to represent samples with a low, medium and high level of plankton, respectively. To each jar a 4% solution of buffered formaldehyde was added. The number of particles per gram wet weight was counted and classified into crustaceans, arrow worms, fish larvae and small medusae. Prior to each egg-sorting experiment, the spray technique was applied enough times to ensure that it was unlikely that the plankton contained any mackerel eggs. Prior to each egg-sorting experiment, 500 mackerel eggs had been counted twice before these were added to the plankton sample.

Procedure of egg sorting by the spray technique

At least 12 h prior to the egg sorting, 500 mackerel eggs were added to the plankton sample, in 4% buffered formaldehyde, which did not contain any eggs. The formaldehyde was rinsed from the plankton sample in a 270 μm mesh sieve with seawater. The plankton was then washed from the sieve into the separating funnel (Fig. 1B) and immediately after it was filled-up with aerated seawater with the spray of the water jet filter pump (Fig. 1A). The spray jet was rotated around, above the floating plankton, to perform an optimal mixing of the plankton with the aerated seawater. Its spray gave the plankton sample a cloudy appearance because of the tiny air bubbles. The sample was then left to stand for ~2 min whilst the aerated plankton floated to the surface and most fish eggs sank to the bottom. By opening the tap, the lower fraction of seawater, containing the fish eggs, was carefully transferred into a 2 L glass beaker. The tap is then closed before the floating plankton can get through. The egg-sorting procedure of filling with aerated seawater, waiting and removing the egg fraction was repeated 10 times to ensure that all 500 mackerel eggs had been retrieved. The 2 L glass beaker was placed in a black tray with water to enhance the visibility of the eggs. Stirring the water caused the eggs and the plankton to concentrate in the middle at the bottom of the glass beaker (Fig. 2). This facilitated the removal of the eggs and plankton particles with a plastic pipette for later counting. Furthermore, the duration of all 10 sorting procedures was recorded; starting when the plankton sample had been transferred into the separating funnel and ending when the egg fraction had been removed after the 10th sorting procedure. During each period of 2 min waiting time, the eggs from the 2 L glass beaker were transferred into vials for subsequent counting.

Procedure of manual egg-sorting method

Exactly 500 mackerel eggs were added to a plankton sample that did not contain any fish eggs. After mixing the eggs with the plankton, the manual egg sorting was

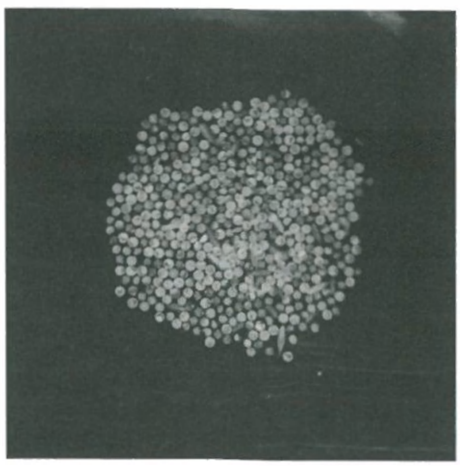

Fig. 2. The first egg-sorting procedure by the spray technique resulted in the highest number of eggs with the lowest number of crustaceans. Stirring concentrated the eggs and crustaceans in the middle of the 2 L glass beaker for easy removal with a plastic pipette. The visibility of the eggs has been enhanced by placing the glass beaker in a black tray with water.

carried out by transferring small aliquots of plankton into an 18 cm diameter Petri dish, which was placed on top of a light screen. A large magnifying glass was used for a better recognition of the eggs. All eggs that were immediately visible were removed with a plastic pipette and transferred into a small Petri dish. Then the sorting started on one side of the dish and gradually continued to the other side. With a plastic pipette, the plankton was moved to that side where the sorting started to indicate the borderline between the area of the dish that was already sorted and that was not yet sorted. This moving of the plankton particles was also carried out to ensure that no eggs remained hidden below or in between plankton particles. During this process, the observed eggs were removed with the plastic pipette and put into the small Petri dish. This procedure was repeated for all aliquots of the total sample. Only one thorough egg sorting of the whole plankton sample took place (no sub-sampling). The duration of egg sorting was recorded; starting after the first aliquot of plankton had been transferred into the Petri dish and ending after the last aliquot of plankton had been checked completely and all eggs had been transferred from the small Petri dish into a vial.

Comparative egg-sorting experiments

The following six egg-sorting experiments were carried out in triplicate in order to enable calculations of precision and bias (accuracy) afterwards (Table I).

In a few instances, the total of counted eggs from the egg sorting did not add up to exactly 500, which might have been due to either a counting error, loss of eggs, egg damage or the plankton had not been completely free of eggs at the start. The predetermined accepted range of the counted total number of eggs at the end of the experiment was 497–503 otherwise the experiment was repeated. The counted numbers have been either raised or lowered in order to add up again to 500 assuming that this was the correct number, because the 500 eggs that were transferred into the plankton sample had been counted twice.

The duration of the 10 sorting procedures by the spray technique and of the thorough manual egg sortings had been recorded. Furthermore, for both egg-sorting methods, the number of crustaceans and other species, which were mixed with the eggs, had been counted to estimate to what extent the egg fraction was contaminated with plankton particles.

Table I: Six egg-sorting experiments were carried out in triplicate for comparing the spray technique and the manual method of egg sorting at three different levels of plankton volume

		Triplicate experiments
SPRAY technique with 10 sorting procedures	1	10 g wet weight of plankton +500 mackerel eggs
	2	40 g wet weight of plankton + 500 mackerel eggs
	3	100 g wet weight of plankton + 500 mackerel eggs
MANUAL method with only one thorough sorting	4	10 g wet weight of plankton + 500 mackerel eggs
	5	40 g wet weight of plankton + 500 mackerel eggs
	6	100 g wet weight of plankton + 500 mackerel eggs

RESULTS

All egg-sorting experiments were carried out with the same set of three plankton samples, which did not differ in species composition, but which only differed in plankton quantity. Consequently, differences in results of egg sorting from these plankton samples can only be attributed to differences in the amount of plankton and therefore enables a good comparison of both egg-sorting methods. The composition of 1 g wet weight of this plankton was estimated as in Table II. The total number of plankton particles per gram wet weight of plankton was 1017. This implied that the control samples of 10, 40 and 100 g plankton, respectively, contained ~10 000, 40 000 and 100 000 plankton particles.

For all three plankton levels, the bias and coefficient of variation (CV) (standard deviation expressed as a percentage of mean) achieved by the first spray procedure was already less than the bias and CV from one thorough egg sorting by the manual egg-sorting method (Fig. 3). Furthermore, the bias and CV decreased rapidly to 0 in the successive egg-sorting procedures indicating that the spray technique appeared to have been able to remove all eggs. Accuracy errors

Table II: All egg-sorting experiments were using plankton samples with the same plankton composition

	Numbers	Percentage
Crustaceans (mainly copepods)	960	94.4
Arrow worms (Chaetognatha)	50	4.9
Fish larvae	2	0.2
Small medusae	5	0.5

The composition of 1 g wet weight of plankton was estimated.

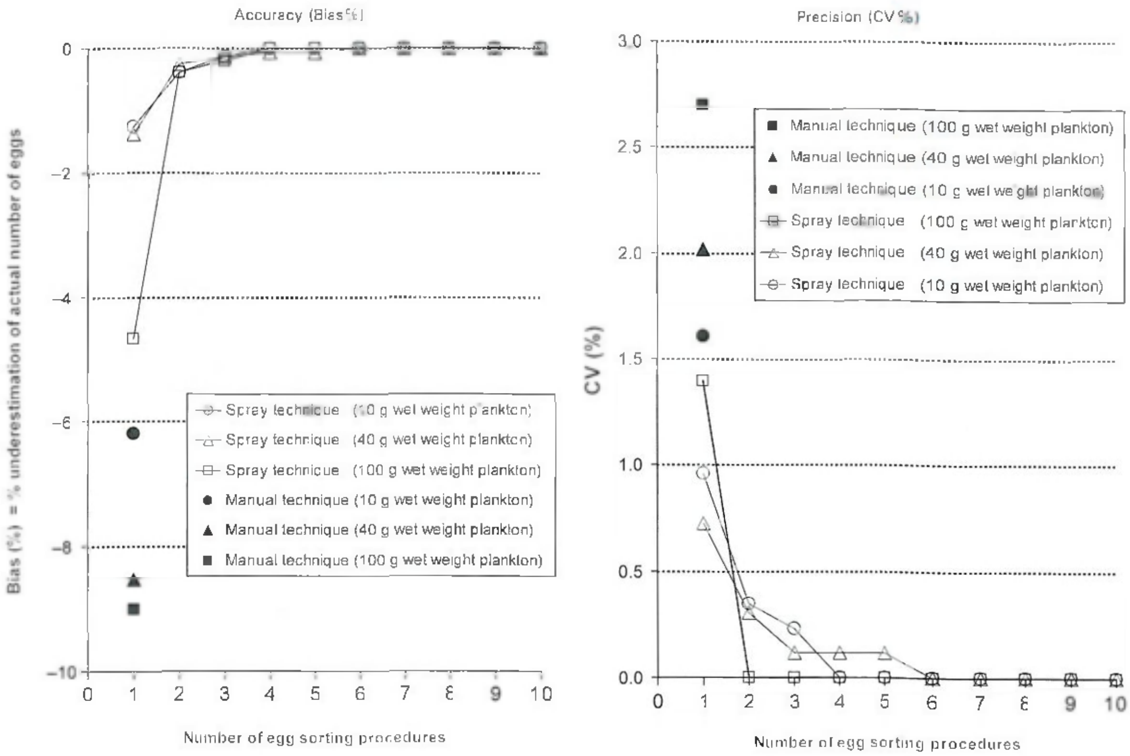

Fig. 3. Bias (accuracy) and CV (precision) have been calculated from triplicate experiments of both the spray technique and the manual egg-sorting technique at three different levels of plankton. These are shown, respectively, in the left and right panel. Accuracy errors correspond to a bias in egg sorting, which can only be an underestimation of the actual total numbers of eggs. Bias is therefore expressed as negative values in percentage. The precision is expressed as CV in percentage. Precision improves with decreasing CV.

correspond to a bias, which can only be an underestimation of the actual total numbers of eggs. Bias is, therefore, expressed as a negative value in percentage.

The collected egg fraction obtained from only the first egg-sorting procedure by the spray method contained 14.2, 4.8 and 5.5 times less contamination with plankton particles than the egg fraction by the manual egg-sorting method for, respectively, the 10, 40 and 100 g plankton samples (Fig. 4). The contamination of the egg fractions with the plankton particles is less than linear at different plankton levels: four times more plankton resulted in a factor of only 1.8 more contamination by plankton particles and 10 times more plankton resulted in a factor of only 2.7 more contamination by plankton particles, when the contamination is calculated over all 10 egg-sorting procedures.

The spray technique appeared to be ~25, 60 and 110 times faster than the traditional manual method when using plankton samples with a 10, 40 and 100 g wet weight of plankton, respectively (Fig. 5). These numbers are based on the duration of only one spray technique procedure compared with duration of the manual egg-sorting method, because the first egg-sorting procedure by the spray technique already achieved a higher accuracy and precision than one thorough manual egg sorting (Fig. 3). Even when applying 10 sorting procedures of the spray technique, the spray method is still 3–11 times faster than the manual method, but achieving then an accuracy and precision of, respectively, 100% of the eggs removed and a CV of 0.0% (Fig. 3). Information on the actual durations of the egg-sorting experiments is provided in Table III.

DISCUSSION

The new spray technique for egg sorting presented in this study is a great improvement compared with the historically developed automatic egg-sorting devices as well as the traditional manual egg-sorting method. It is very fast and efficient. It is extensively compared to the traditional manual method and its advantages and disadvantages are discussed and evaluated later.

Historically developed automatic egg-sorting devices

Bowen *et al.* (Bowen *et al.*, 1972) described that fish eggs and larvae can be separated from invertebrate

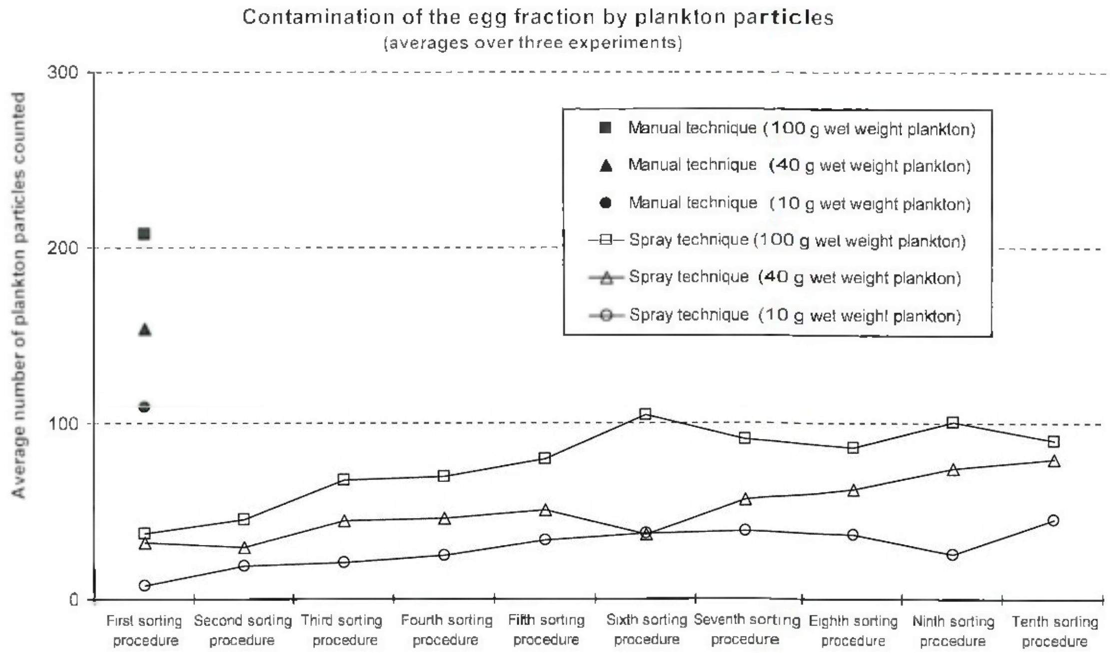

Fig. 4. The average contamination of the egg fraction by plankton particles by egg-sorting procedure of the spray technique and for one thorough sorting by the manual egg-sorting method as estimated from egg-sorting experiments with low, medium and high plankton levels.

zooplankton by isopycnic centrifugation in gradients of sucrose and silica. Of the six dominant classes of zooplankton, only Salpa overlapped with the fish eggs and none overlapped with the fish larvae. Plankton sample size was 1 g wet weight; 10 g was maximum size for this method. They stated: "This system could substitute for the hours of laborious handwork presently necessary for ichthyoplankton sorting, and it would require a minimum of operator time, readily available centrifuge equipment, and inexpensive gradient materials" and "plankton sorting by gradient centrifugation will not be practical for samples collected during *Phaeocystis* blooms until some method is devised either to break down or to remove the filaments". There is no direct estimate of

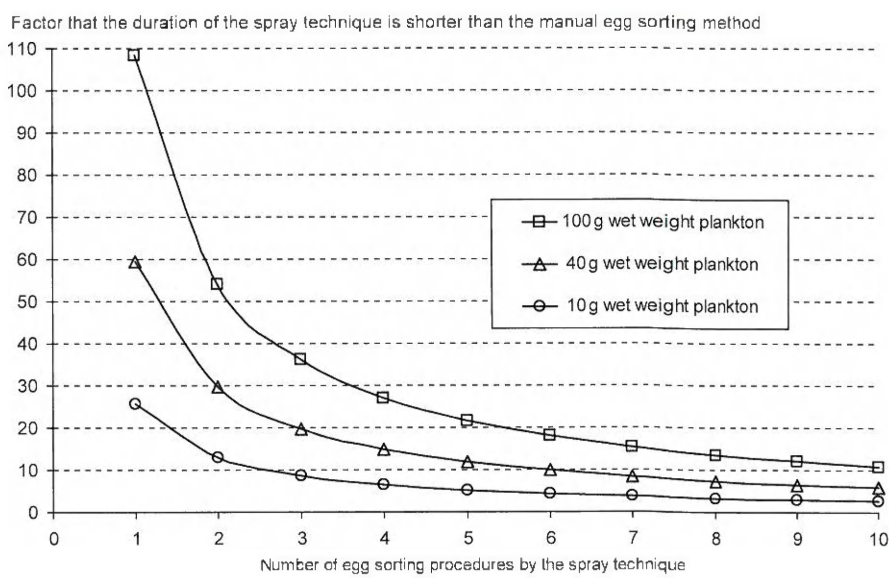

Fig. 5. Indication of how much faster the spray technique is compared with the manual egg-sorting method for three different levels of plankton wet weight.

Table III: Durations of egg sorting by the traditional method of manual sorting for only one thorough sorting and by the new spray technique presented from 1 to 10 spray procedures

	Duration of egg sorting (h:min:sec)											
	10 g wet weight plankton				40 g wet weight plankton				100 g wet weight plankton			
	A	B	C	Mean	A	B	C	Mean	A	B	C	Mean
Manual technique	1:19:45	1:12:36	1:01:50	1:11:24	2:43:46	2:43:09	2:39:30	2:42:08	5:13:09	4:01:09	5:08:52	4:47:43
1 Spray procedure	0:02:51	0:02:39	0:02:49	0:02:46	0:02:46	0:02:45	0:02:41	0:02:44	0:02:39	0:02:39	0:02:40	0:02:39
2 Spray procedures	0:05:41	0:05:19	0:05:38	0:05:33	0:05:31	0:05:29	0:05:23	0:05:28	0:05:17	0:05:18	0:05:21	0:05:19
3 Spray procedures	0:08:31	0:07:58	0:08:27	0:08:19	0:08:16	0:08:14	0:08:04	0:08:11	0:07:56	0:07:57	0:08:01	0:07:58
4 Spray procedures	0:11:22	0:10:38	0:11:16	0:11:05	0:11:02	0:10:59	0:10:45	0:10:55	0:10:34	0:10:36	0:10:42	0:10:37
5 Spray procedures	0:14:13	0:13:17	0:14:05	0:13:52	0:13:47	0:13:43	0:13:26	0:13:39	0:13:13	0:13:15	0:13:22	0:13:17
6 Spray procedures	0:17:03	0:15:57	0:16:54	0:16:38	0:16:33	0:16:28	0:16:08	0:16:23	0:15:52	0:15:53	0:16:03	0:15:56
7 Spray procedures	0:19:53	0:18:37	0:19:43	0:19:24	0:19:18	0:19:13	0:18:49	0:19:07	0:18:30	0:18:32	0:18:43	0:18:35
8 Spray procedures	0:22:44	0:21:16	0:22:32	0:22:11	0:22:04	0:21:58	0:21:30	0:21:51	0:21:09	0:21:11	0:21:24	0:21:15
9 Spray procedures	0:25:35	0:23:55	0:25:21	0:24:57	0:24:49	0:24:42	0:24:12	0:24:35	0:23:47	0:23:50	0:24:05	0:23:54
10 Spray procedures	0:28:25	0:26:35	0:28:10	0:27:43	0:27:35	0:27:27	0:26:53	0:27:18	0:26:26	0:26:29	0:26:45	0:26:33

efficiency of the method compared with the manual egg-sorting method, and the amount of plankton contamination in the collected egg fractions is unknown.

Price *et al.* (Price *et al.*, 1977) described an automatic sorting of zooplankton by isopycnic sedimentation in gradients of silica sol. Following an overnight period of sedimentation and equilibrium, the fish eggs were recovered from the top of the gradient, invertebrates from the central part of the gradient and fish larvae from the bottom of the gradient. *Phaeocystis* spp. and hydroids renders the plankton samples unsuitable for separation by sedimentation (Price *et al.*, 1977). This study was initiated in an effort to reduce the sorting time for plankton samples. The authors only mentioned: "the system can appreciably reduce sorting time". However, it is not stated to what extent.

Mamhot *et al.* (Mamhot *et al.*, 1988) described zooplankton sorting by sedimentation in gradient solutions as a method for rapid separation of fish larvae. It greatly reduced the time of all manual sorting by about one-half. However, the fish eggs did not constantly sediment in any fixed layer. It seems therefore appropriate to conclude that this method does not appear to be useful for automatic egg sorting.

Although there have been major advances in the use of image analysis and/or particle counters for the analysis of plankton (Wiebe and Benfield, 2003; Culverhouse *et al.*, 2006), none of the methods reported allow fish eggs to be automatically identified by species and staged. Thus manual egg enumeration is still required. No references could be found on image analysis and/or particle counters related to egg-sorting devices.

Spray technique in comparison to the manual egg-sorting method

There are many advantages of the spray method and these are listed below in an overview and then further discussed in more detail. Results from the comparative experiments demonstrated that the spray method is:

(i) extremely fast compared with the traditional manual egg-sorting method (Fig. 5 and Table III);
(ii) as accurate and precise as required, because it can easily be repeated as often as necessary (Fig. 3);
(iii) excellent in separating the plankton from the fish eggs (Fig. 4);
(iv) performs well up to high levels of plankton (Figs 3 and 4);

In addition the spray method has the following important advantages:

(v) Much less prone to "human errors" than the manual egg-sorting method;
(vi) It can be standardised;

Additional advantages based on experience obtained during the mackerel egg surveys:

(vii) It performs well onboard research vessels and appears to cope with different species compositions of the plankton.

The spray method did not appear to have any disadvantages compared with the manual egg-sorting method regarding the egg sorting. However, the spray technique does not work for the removal of fish larvae, whereas the manual method does. Furthermore, future users of the spray technique should be aware that the spray technique should not be used too soon after having added 4% formaldehyde to the plankton sample, because the buoyancy of the eggs appeared to increase only gradually over time due to fixation. This important possible pitfall when starting to use the spray method is discussed later in more detail. In addition, the spray method worked well with mackerel eggs and is likely to function well for other fish eggs with a hard and firm chorion. However, problems in applying the spray technique might occur with fish eggs with a soft chorion (e.g. sardine). This problem has not been investigated in this study.

The spray technique is a very fast method, because each sorting procedure takes only less than 3 min (Table III). Therefore, it can be repeated easily until a required accuracy and precision is achieved. It appeared to be approximately a factor 25, 60 and 110 faster than the traditional manual method when using plankton samples with, respectively, a low, medium and high plankton level. These factors are based on the duration of only one spray technique procedure compared with the duration of the manual egg-sorting method, because the first egg-sorting procedure by the spray technique already achieved a higher accuracy and precision than one thorough manual egg sorting (Fig. 3). It should be noted that these factors should be regarded as only indicative (see also comments on standardisation of the spray method).

During the last international ICES Mackerel and Horse Mackerel Egg Surveys in 2004, eight countries collected during 291 ship days 1760 plankton samples (ICES, 2005). A very rough indication of the reduction in egg-sorting time for this survey might be ~2600–3500 man-hours assuming that the new spray technique reduces on average the sorting time by 1.5–2 h per plankton sample (the wet weights of the plankton samples are not available to allow a more accurate estimation).

The spray technique can be repeated easily, because it is a very fast method of which each sorting procedure takes only less than 3 min. This allows, therefore, a continuation until a high accuracy and precision is achieved (Fig. 3 and see also comments on standardisation of the spray method).

The experiments have demonstrated that the collected egg fraction obtained from the first egg-sorting procedure by the spray method contained much less contamination by the plankton particles than the egg fraction by the manual egg-sorting method (Figs 2 and 4). The contamination of the egg fractions with the plankton particles appeared to be less than linear at different plankton levels. The first egg-sorting procedures of the triplicate experiments with the 100 g wet weight plankton showed that on average only 38 plankton particles were mixed with the eggs while the plankton samples contained ~100 000 plankton particles. This represents an excellent separation of the plankton from the fish eggs, because in these 100 g wet weight plankton samples, only 0.038% (in numbers) of the total plankton particles mixed with the eggs, whereas 99.962% (in numbers) was retrieved in the floating layer. During successive egg-sorting procedures, the contamination by plankton particles gradually increased but always remained lower than the contamination estimated from the manual egg-sorting method.

The experiments demonstrated that the spray method performs well up to a high plankton level of 100 g wet weight plankton. Just if there appears to be too much plankton in one sample, the plankton sample can always be split into two aliquots for spraying.

Up to now, various institutes have applied the traditional manual egg-sorting technique. The accuracy of it may depend on the procedures used, e.g. whether the eggs were observed by eyesight or by binocular microscopes/magnifying glasses, whether sub-sampling was applied to reduce sorting time, etc. However, the person who actually is carrying out the manual egg sorting is expected to determine the achieved accuracy to a much larger extent. These so-called "human errors" probably have a significant effect on the accuracy. These are: the time taken or the time available for egg sorting or the speed of egg sorting, the ability to work accurately, the quality of the eyesight, the ability to recognise round shapes easily in between the many plankton particles, the ability to concentrate for a long time, the size of the plankton aliquots, etc. This implies that an accuracy and precision figure of the traditional manual egg-sorting method cannot be given, because it depends too much on these errors. In addition, the accuracy is expected to decrease with an increasing volume of plankton in the sample.

In this study, the accuracy and precision in manual egg sorting have been estimated for the author of this study, who carried out these egg-sorting experiments. The accuracy achieved might be an underestimate, since the author is not an experienced manual egg sorter. The triplicate manual egg-sorting experiments with the 100 g wet weight plankton samples indicated that the egg-sorting duration, which is related to plankton aliquot size, has a large impact on the accuracy. In 4 h of egg sorting, only 88% of the eggs could be removed, whereas in 5 h, 92% of the eggs could be extracted. This indicated that at least 6 or 7 h egg sorting might probably be needed to achieve 95% retrieval as was already achieved for only one egg-sorting procedure by the spray technique within less than 3 min. Therefore, the estimation of how much faster the spray technique is compared with the manual egg-sorting method may be an underestimate, though, perhaps, compensating for the fact that the author is not an experienced manual egg sorter.

It is important to note that the proportion of eggs removed per egg-sorting procedure by the spray technique probably depends on:

(i) the specific density of the eggs, which differs by fish species and by egg developmental stage (Coombs, 1981; Coombs *et al.*, 1985) and furthermore is expected to change over time after fixation with 4% formaldehyde;
(ii) the amount of plankton as well as the species composition of the plankton;
(iii) the salinity of the seawater used for spraying;
(iv) the degree of aeration of the seawater for spraying.

Therefore, the presented results of this study should not be regarded as being representative for other applications of the spray technique. In other cases, it might be that more egg-sorting procedures are needed to remove all eggs. "**It is recommended to a target accuracy in egg sorting in advance of the actual egg sorting**" (e.g. bias should be less than −2%, which corresponds to a removal at least 98% of the eggs from a plankton sample). Furthermore "**it is recommended to follow a standard operation procedure, when applying the spray technique**" (e.g. apply the spray technique to each plankton sample at least three times and continue to do so until three times in succession no more eggs are found). Such a standard operation procedure might help to ensure that the target accuracy in egg sorting will be reached. The knowledge of how many eggs are removed during the last egg-sorting procedure is one of the major advantages of the spray method, because it allows an immediate decision to continue or to stop.

It should be emphasised that whenever the spray technique is applied, the accuracy should be estimated at regular intervals to evaluate whether the target accuracy in egg sorting has been achieved. Plankton samples that contain large numbers of eggs are especially suitable for this purpose. First remove all eggs from a certain plankton sample according to the agreed standard operation procedure and then afterwards remove all remaining eggs from this sample (by the manual egg-sorting method and/or by applying the spray technique as many times as possible). The accuracy (bias) in egg sorting can be estimated as follows:

Bias (%) = −100 × [(eggs counted afterwards)/(counted eggs from standard operation procedure and eggs counted afterwards)].

The standard operation procedure for the spray technique should be revised, if the targeted accuracy in egg sorting has not been achieved or overshot too much. This strategy can provide a standardised method, which can be applied by different users and ensures similar accuracies and precision levels of the egg-sorting method and excludes most of the possible errors that apply to the manual egg-sorting method.

The separating funnel should not be shaken or disturbed when the eggs separate from the floating plankton otherwise part of the floating plankton might sink and mix with the eggs. However, this separation technique is sufficiently robust that it can successfully be applied on board research vessels during ichthyoplankton surveys. The slow movements of the ship and the ships vibrations did not seem to increase the plankton sedimentation.

The egg-sorting experiments demonstrated that the spray technique performed well with plankton consisting out of mainly crustaceans and Chaetognatha (arrow worms). Additionally, based on authors experience in applying the spray technique during the egg surveys carried out from 2001 to 2005, the spray method appeared to cope quite well with other plankton species such as: small jelly-fish, Ctenophores, salps, pelagic snails, phytoplankton, etc. In manual egg sorting, large amounts of phytoplankton can cause a problem, because eggs can become entangled in it. The spray technique is more successful in removing eggs from this relatively dense phytoplankton, because the powerful spray of aerated seawater can disentangle the eggs from it during successive spray procedures.

Before the actual egg sorting can take place, both the traditional manual method and the new spray technique require a certain waiting time after having added 4% formaldehyde fixative to the fresh caught plankton sample. Fresh eggs are difficult to remove by eyesight with a pipette because of their translucency, which makes them more or less invisible in seawater. Therefore, the plankton samples should be left in a 4% formaldehyde solution long enough for the fish eggs to become visible (opaque). However, regarding the spray technique, the waiting time should be even more prolonged because fixation should in addition increase the specific density of the eggs. The importance of the latter was experienced during the 2001 mackerel and horse mackerel egg survey.

Onboard RV "Tridens", the fish eggs were removed by the spray technique, while the 4% formaldehyde fixation time was limited to the time in between two plankton stations, which corresponded to ~2 h. Back at the laboratory, these plankton samples were again sorted and the eggs staged. Only half of all the mackerel and horse mackerel eggs were removed onboard, but it was remarkable that these onboard sorted eggs consisted of over 60% stage 1 eggs, whereas the other half of the eggs, which were removed at the laboratory, consisted of only ~30% stage 1 eggs (Ingeborg de Boois, IJmuiden, personal communication). According to Coombs (Coombs, 1981), there is a progressive declining trend in the specific density (from 1.0245 to 1.0205 g cm^{-3}) of fresh mackerel eggs during egg development. This is the likely explanation why mainly stage 1 eggs with a relatively higher specific density were removed onboard by the spray technique and that the older egg stages still remained in the plankton sample. Coombs (Coombs, 1981) further noted that moribund mackerel eggs sank to the bottom. The specific density at the bottom of the density-gradient column was 1.040, which implies that moribund mackerel eggs appear to have a specific density larger than 1.040 g cm^{-3}.

On the basis of the above-described experience, it can be concluded that the increase in specific density of eggs that have been put in 4% formaldehyde is a relatively slow process. No experiments have been carried out, however, to evaluate this process, but a strategy has been applied to wait with the spray technique at least 24 h after the onset of fixation. Later checking for remaining eggs in the plankton at the laboratory confirmed that this strategy worked quite well. However, it is still possible that the efficiency of the spray method might even increase when the fixation time is further extended than 24 h.

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