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Commerical preparations and published formulations using reagent chemicals for seawater substitutes were not biologically acceptable to Mytilus edulis embryos, in that calcification did not occur. When Leslie coarse hide salt was used as the source of NaCl for literature formulations, most formulas provided some success in the development, growth and calcification of mussel embryos.

By varying concentrations of several anions and cations, and using Leslie coarse hide salt, a new formula (BioSea) was developed which would allow normal growth and development to the shelled veliger stage. Essential inorganic compounds for M. edulis development are NaCl; $NaHCO_3$, KCl, anhydrous $MgSO_4$ and $CaCl_2$. The Leslie coarse hide salt (NaCl) used in BioSea contains an essential compound or compounds (L factor), thought to be organic, necessary

for calcification of mussel larvae. This factor is destroyed upon burning of the salt. Seawater contains 4 times as much of the essential L factor as BioSea. A solution of 20% real seawater, with the salinity adjusted to 25 ppt using reagent NaCl will provide all necessary compounds for normal development of M. edulis. Growth and development of Mytilus was improved by adding alkaline phosphatase, carbonic anhydrase, purine (free base), DL lysine and DL aspartic acid to BioSea.

Abnormal larvae could often be related to specific substances or specific procedures. Nine abnormalities of frequent occurrence were described. These included hollow embryos, ear muff, black spot and exploded types, levorotory spin, malformed trochophores, trocophores without flagella and larvae with extrusion of viscera.

Results of mussel bioassays with Kraft mill effluents in BioSea were equivalent to those using natural seawater. It is suggested that BioSea be used as standard media for bioassays of marine waters. Additional research is strongly recommended towards identifying the L factor (Leslie Coarse hide salt factor) which is necesdary for calcification of shellfish larvae.

FORMULATION OF A SYNTHETIC SEAWATER FOR BIOASSAYS WITH *MYTILUS EDULIS* EMBRYOS

by

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INTRODUCTION

The wastes of mankind have reached magnitudes which create intense pollution in our bays, estuaries and marine environments. Organisms living there have specific requirements for their well-being and will reflect the effects of various toxic wastes in dissimilar fashions. Useful parameters, standards and processes for waste treatment and abatement in seawater have not been developed. Involved in this failure are the variability of seawater itself, the variability between synthetic mixtures that have been used as substitutes for a marine environment and apparent differences in experimental findings, due in a large extent, to inadequate information about the sea and the organisms in it.

Major problems confronting marine biologists and toxicologists are the uncontrollable changes in seawater quality. These changes are particularly evident in biological systems where toxicity bioassays are involved. The marine investigator is concerned with the vastly intricate and dynamically complex chemical and biological properties of the ocean, without the aid of a satisfactory chemically known control solution. Freshwater biologists control their experiments with pure distilled water or natural waters of known chemical

characteristics. Unfortunately however, present artificial seawater formulations will not allow higher life forms to complete their life cycles. This is typically true for shellfish, as the synthetic media are either toxic or lack the essential components for successful gametogensis and calcification. The need for a reliable and reproducible synthetic seawater is a prime necessity for the evaluation of modern pollution problems.

Embryos of the bay mussel Mytilus edulis have been used by marine pollution biologists (Dimick and Breese 1965) to compare and monitor the toxicity of pulp mill effluents. But a standard synthetic seawater which was biologically acceptable to the mussel embryo and chemically suitable as a control water in marine toxicological investigations was not available. A standard and reliable synthetic seawater is also needed to culture pure strains of marine algae and to provide a physical basis for physiological, embryological and cytological experimentation. The purpose of this study was to develop a synthetic seawater specifically for bioassay use with the Mytilus edulis embryo. The formulation which appeared to be best for this purpose is referred to as BioSea.

LITERATURE REVIEW

Seawater analyses by many investigators (Sverdrup, Johnson, and Fleming 1946; Harvey 1928, 1955, 1957; and Thompson and Robinson 1932) have shown that the inorganic macro-constituents of the oceans are of surprisingly uniform concentrations through throughout the world. This chemical uniformity of the sea led Coker (1947) to suggest that the sea is an inexhaustible reservoir of every chemical necessary for life and they are in such quantities that if the whole of mankind took its entire chemical supply from the ocean, there would not be the slightest detectable change in the composition of the sea. However, most of the inorganic substances are in the ionized form and are susceptible to diverse recombination with other organic and inorganic molecules. Wilson (1951) asserts that many of these ions, such as silicate for diatoms and certain sponges, calcium for the shells of molluscs and vanadium for the blood of some tunicates, are biologically utilized in various ways by organisms with specific needs. Each organism has inherent genetic differences, requirements and uses for the compounds in the medium which surrounds it, and biologically speaking the sea is not uniform, but is as diverse as the creatures which inhabit it.

Recently, research has been directed towards the organic compounds of the ocean. Various investigators (Bader, Hood and Smith

1960; Slowey, Jeffery and Hood 1962; Tatsumoto 1961; Anderson 1939) have shown that there is one to five milligrams of organic material per liter in the seawater. Wangersky (1952) discovered rhamnoside and dehydroascorbic acid; Hargreaves, Agrahams and Vickery (1951) reported citric and malic acids; and Cowey (1956) found vitamin B_{12}. In 1958, Smith demonstrated that CO_2 in seawater is affected by carbamino carboxylic acid complexes and the solubility of calcium carbonate is affected by dissolved organic matter in the sea.

Many other organic compounds in seawater and sea sediments have been identified by Degens, Reuter and Shaw (1964). Their analysis of amino acids showed concentrations from 16 to 125 micrograms per liter of different acids. Some of the free amino acids were not present in true ionic solution, but were loosely attached to other constituents. Arginine, lysine and beta-alanine made up 40% of the amino acids in sea sediments, but were lacking in seawater. Ornithine and serine made up approximately 40% of the free and combined amino acids in seawater, but accounted for less than 5% in the sediments. This was attributed to the fact that many organisms are capable of producing arginine from ornithine and aspartic acid by way of citrulline and arginine succinic acid in the urea cycle. Lysine could be formed de novo by microbial synthesis. Beta-alanine could be provided by metabolic decarboxylation of

aspartic acid, one of the dominating amino acids in the overlying waters. The amino acids identified in surface seawaters were: arginine, ornithine, histidine, lysine, glutamic acid, aspartic acid, glycine, serine, valine, alpha-alanine, leucine, threonine, proline, tyrosine, phenylalanine and methionine. Also identified were free sugars in concentrations of 14 to 135 micrograms per liter. These in decreasing order of abundance were glucose, mannose, galactose, rhamnose and arabinose. Two amino sugars, glucosamine and galactosamine and some phenols were also present.

Organisms living in a solution of such a heterogenous composition may select and utilize specific ions and compounds for their well being. Allen (1914) reported in his classic experiment on diatom growth, the need for an unknown organic constituent in the water. He experimented with Thalassiosira gravida in artificial seawater and showed that it thrived in the presence of an unknown substance of organic origin. This was one of the first recorded experiments which directly related the uptake of organic material by an organism.

Collier, Ray and Magnitzky (1950;1953) noted that naturally occurring organic substances in seawater affected the feeding of oysters and suspected a carbohydrate or a compound quantitatively associated with carbohydrates. This conclusion was based on the observed correlation between the pumping rates of the oysters and a hitherto unreported factor naturally found in seawater. This factor

gave a positive reaction with the N-ethyl-carbazole reagent test, which is used to quantitatively determine minute amounts of carbohydrates. Loosanoff (1963) reported that unidentified substances in seawater were necessary for normal development and calcification of shellfish. In 1966 Tyler, Piatigorsky and Ozaki, by using C^{12} and C^{14} labeled amino acids, demonstrated that fertilized and unfertilized sea urchin eggs incorporate amino acids from the surrounding medium into the protein of the egg. Some amino acids were incorporated into the eggs more readily than others and unfertilized eggs did not allow the transfer of amino acids as well as fertilized eggs.

Yonge, cited in (Wilbur and Yonge 1964) noted some of the organic compounds which contribute to the osmotic concentration in the cells of M. edulis; these included various amino acids and trimethylamine oxide. In addition he suggested that other organic compounds in solution serve as sources of energy, growth stimulators and complexing materials but some may act also as toxins.

The literature contains evidence that organisms utilize dissolved chemicals in seawater for their metabolism, growth and development. Therefore it is necessary to ascertain the specific materials which are required by the organism of interest, if one desires to formulate a biologically acceptable substitute water for that organism. The seawater to be developed was to be used in bioassays employing shellfish. Since the end point on which

satisfactory performance was to be based, was a normal, straight hinged shelled larvae, as opposed to a larvae with no shell at all, it is necessary to review the literature concerning calcification and shell formation in molluscs.

Bevelander (1952) describes two distinct phases during molluscan calcification: First, the formation of a protein membrane (periostracum) and second, the mineralization of this material to form the shell. There are several concepts which attempt to explain calcification in molluscs; unfortunately many factors are subject to conjecture and speculation.

Bevelander and Benzer (1948) state that the mucous glands covering the three folds in the mantle epithelium of bivalves contain many intracellular granules. These granules give a positive test for calcium phosphate. Also, that during the process of calcification the free edges of the mantle undulate back and forth secreting a fibrous organic material with the simultaneous concentration and deposition of mineral salts. The organic component is apparently a protein which is associated with one or more reducing sugars. This protein has been given the name of conchiolin by Wetzel (1900) cited in Field (1922). Immediately after the protein is formed, calcium phosphate granules are deposited on it and undergo crystal growth. As the crystals enlarge, they assume a polyhedral shape enclosed in the organic matrix. However, the final shell is composed largely of

calcium carbonate, with traces of aragonite, the orthorhombic allomorph of calcium carbonate. It was suggested that the large concentrations of the enzyme alkaline phosphatase which was detected in the middle fold of the mantle epithelium, may aid in the transfer of calcium phosphate to calcium carbonate. This mechanism, possibly an oxidation-reduction reaction, is not clearly understood (Bevelander 1952; Hirata 1953).

The carbonic anhydrase content was determined in twenty species of molluscs by Freeman and Wilbur (1948) using a cytochemical procedure outlined by Ferguson (1937). This enzyme functions as a catalyst in the hydration of CO_2 and it is thought to be associated with the metabolic portion of CO_2 in the carbonate of molluscan shells. The enzyme is found in the mantle edge and throughout the body of the mantle in most molluscs. Galtsoff (1934) showed that shell formation in the oyster proceeds during hibernation at temperatures below 4° centigrade and concluded that the action of carbonic anhydrase becomes more important with a decrease in temperature.

Other mantle enzymes were studied by Jodrey and Wilbur (1955). They reported the presence of isocitric, succinic and malic dehydrogenases, fumarase, and cytochrome oxidase in the mantle of Crassostrea virginica. Oxaloacetic decarboxylase was also found in high concentrations and therefore believed to be important in the

formation of shell carbonate. It is thought that respiration may be involved with the transport of organic and inorganic materials through the cells of the mantle tissue, because CO_2 from respiratory intermediates could provide a metabolic source for the carbonate in the shell.

Tricalciumcitrate, $Ca_3 (C_6H_5O_7)_2 + 4H_2O$, may play a part in the calcification process (Steinhardt, 1946). He has made an analysis for citric acid, calcium, and phosphoric acid in bird and crocodile eggs and in marine corals and oyster shells. Oyster shells contained 17 mg citric acid, 19 mg phosphoric acid and 32.8 grams of calcium per 100 grams of shell.

The amino acid content in the protein from the aragonite and calcite in the shells of Mytilus californianus was studied by Hare (1962; 1963). He noted high glycine residues in the prismatic calcite and nacreous (mother of pearl) aragonite layers of the shell.

Turekian and Armstrong (1960) found traces of Mg, Sr, and Ba associated with the calcium carbonate in molluscan shells. The amounts are peculiar to each species and depend upon the microenvironment of the animal. Wetzel (1900) cited in Field (1922), reported that the shell of M. edulis contained Ca, Mg, Fe, Mn, SiO_3, SO_4, PO_4, Cl and sulfides. In addition, the decomposition products resulting from H_2SO_4 oxidation of the organic conchiolin were, glycocoll, leucin and tyrosine. He classified conchiolin as between

casein and egg albumin.

Many investigators have used synthetic seawater for growing pure cultures of algae and diatoms (Hutner 1948; Pinter and Provasoli 1958; Droop 1960; Provasoli and McLachlan and Droop 1957; McLachlan 1964; Chu 1942; Squros and Simms 1963). The basic formulations are similar, but the needs of algae for different trace elements and vitamins make the mixtures toxic to embryonic forms of higher marine animals.

The investigation here reported indicates that synthetic saltwaters designed by other investigators, cited in Lutz, Galtsoff and Needham (1937), were (McClendon 1917; Brujewicz 1931; Lymans and Fleming 1940; Herbst 1903); and commercial saltwaters such as Rila Marine Mix, Instant Ocean, Magic Sea Salts and so on, are unstable or noncompatible with growth and development of embryonic bivalve molluscs when U.S.P. or reagent grade chemicals were used in their preparation.

MATERIALS AND METHODS

Synthetic saltwater formulations described in the literature and available commercial preparations were tested as culture media for the embryos of the bay mussel Mytilus edulis. Additional formulations developed at the Oregon State University Marine Science Center were also studied.

Biological Materials and Methods

Adult mussels were collected from pilings and docks in Yaquina Bay, Newport, Oregon. Gametes from the mussels were obtained by chemical stimulation with 0.2M potassium chloride in seawater of 25 ppt salinity. Gametes were placed in 250 ml round test vessels containing seawater or a synthetic substitute. These were maintained at 20° Centigrade. Approximately 5000 embryos were used per 100 ml of test seawater. The test vessels were covered to prevent evaporation and held at room temperature during the 48-hour incubation period. All natural and artificial seawaters were adjusted to a salinity of 25 ppt with distilled water before testing. The primary criterion of effectiveness in all tests was the percent normal shelled larvae of the first 150 larvae counted under a dissecting microscope in a 30 ml aliquot of test solution. The foregoing mussel bioassay procedure is discussed in detail by Dimick and Breese

(1965). At least two females were involved in each comparison and at least two test vessels were used per formulation per female.

Organic compounds and combinations thereof were recorded as effective, possible, non-effective or detrimental when related to the number of normal embryos developing and also to embryo size, normal shell calcification and locomotion of the veligers. Locomotion of the larvae was visually estimated by comparison with the control and recorded as very active, active and moderate.

Monochrysis sp. algae, for feeding Mytilus veligers were grown in BioSea 14 with multivitamin and nitrate additives according to a formulation by John G. Denison of the Rayonier Marine Laboratory in Hoodsport, Washington.

Chemical Materials and Methods

The water used to make up synthetic solutions was glass distilled but natural spring and rain waters were also tried. Samples of seawater were obtained from Yaquina Bay, Newport, Oregon.

Different grades of chemicals and sodium chloride from various natural and commercial sources were evaluated as to their biological acceptability to the mussel embryo. Trace elements were used in concentrations which approximated those occurring in natural seawater as listed in the Hand Book of Chemistry and Physics (1965). Concentrations of organic compounds used were

0.033 mg/100 ml, 0.00033 mg/100 ml and 0.000033 mg/100 ml respectively. Stock solutions of organic compounds were placed in stoppered 300 ml bottles and refrigerated in the dark at 4^{o} Centigrade.

Strong wastes from the Kraft pulp mill at Toledo, Oregon were used during the comparative toxicity studies between BioSea and natural seawater. These wastes contain about 10% fiber, Na_2SO_4, various lime muds, many dissolved organic materials and water. The color is usually dark brown and the waste has a pH of about 11.

The facilities of the O. S. U. Marine Science Center, Pacific Fisheries Laboratory, Newport, Oregon were utilized during the major portion of this study.

DISCUSSION AND INTERPRETATION OF DATA

Artificial Seawater Formulations from Literature

The logical first step in searching for a suitable artificial seawater for bioassay use with embryos of M. edulis was to test formulations by other investigators. The ten formulations in Appendix A were tested and in every case, only abnormal larvae were obtained. It is not known if the formulators experimented with M. edulis or if they studied other embryonic forms of life in their synthetic mixtures. Most formulations were designed for maintaining adult forms of marine animals and therefore criticism of the formulas tested is not offered here. However, they are not suitable for use with embryos of M. edulis.

Some possibilities for the failure of the M. edulis embryos to develop normally were that the chemicals used in the formulations were of the wrong quality or quantity or that some essential compounds were lacking. Therefore, tests were started to determine the role of specific salts. The simple Brujewicz formula was used as a starting point for testing different chemical grades of NaCl and also water from various sources (Appendix B). It was discovered that when Leslie coarse hide salt was used as the NaCl in the Brujewicz formula, some embryos developed to normal veligers. But, the

larvae were small and poorly shaped in comparison with those in real seawater. Other grades of NaCl, when used in the Brujewicz formula, did not yield veligers. It was then decided to try to design a synthetic seawater (BioSea) especially for M. edulis and if possible, to determine why the Leslie coarse NaCl would foster calcification of shellfish when other grades of NaCl did not.

Inorganic Requirements for Mytilus edulis

Table 1 shows the macro-inorganic compounds essential for developing M. edulis embryos. These are Leslie coarse rock salt as the NaCl source, U.S.P. grade $NaHCO_3$ and KCl, reagent grade anhydrous $MgSO_4$ and $CaCl_2$. Formulation 14 was considered to be the best combination of these salts as many normal larvae developed when it was used. Formulation 14 has been successfully used in over a hundred tests.

It was noted that fertilization and subsequent polar bodies on the eggs did not occur until $MgSO_4$ was added. Of particular interest is the unexplained fact that $MgSO_4$ must be used in the anhydrous form to achieve normal shelled larvae. Furthermore, complete calcification cannot occur unless Ca is in the form of $CaCl_2$. A list of other calcium salts and trace elements which were tested in BioSea 14 and evaluated as non-effective or detrimental to the embryo are found in Appendix C.

Table 1. BioSea formulations in grams/liter. Salinity subsequently adjusted to 25 ppt with distilled water.

Number of tests*	BioSea #	Leslie coarse NaCl	$NaHCO_3$	KCl	$MgSO_4$	$CaCl_2$	Other	Normal M. edulis veligers
6	A	25.0	---	---		---	---	0
6	B	25.0	0.2	---		---	---	0
6	C	25.0	0.2	1.0		---	---	0
6	D	25.0	0.2	1.0	3.0 Anhy.	---	---	0
6	E	25.0	0.2	1.0	3.0 Anhy.	0.5	---	Poor
10	1	25.0	0.2	0.5	3.0 Anhy.	0.6	**Polyopterin	Fair
3	2	50.0	0.4	1.0	2.0 Anhy.	1.0	---	0
4	3	26.0	0.2	0.5	1.0 Anhy.	0.5	---	0
10	4	33.0	0.2	0.5	12.5 $7H_2O$	1.0	---	Poor
10	5	33.0	0.2	0.5	6.83 $7H_2O$	1.0	---	Poor
6	6	33.0	0.2	0.5	1.0 $7H_2O$	1.0	---	0
4	7	25.0	0.2	0.5	1.0 $7H_2O$	0.5	---	0
6	8	33.0	0.2	0.5	5.75 Anhy.	1.0	---	Fair
6	9	33.0	0.2	0.5	3.3 Anhy.	1.0	---	Fair
6	10	33.0	0.2	0.5	3.3 Anhy.	0.5	---	Fair
4	11	30.0	0.2	0.5	3.3 Anhy.	1.0	11.3 $MgCl_2.6H_2O$	0
10	12	33.0	0.2	0.5	5.75 Anhy.	0.5	---	Fair
12	13	33.0	0.2	0.5	4.25 Anhy.	1.0	---	Good
100	14	33.0	0.2	0.5	3.75 Anhy.	1.0	---	Excellent
4	15	32.0	0.2	0.5	3.75 Anhy.	1.0	1.0 Morton's Iodized NaCl	0
4	16	26.5	0.3	0.73	2.0 $7H_2O$	1.2	---	0
4	17	25.0	0.2	0.6	1.5 $7H_2O$	0.6	---	0
4	18	26.5	0.2	0.73	3.3 $7H_2O$	---	$MgCl_2$ 2.5 $CaCO_3$ 1.5	0

* Tests run in duplicate with each of two or more females.

** Polyopterin -- An impure complex of amino acids and other organic compounds produced by dissolving Sebastoes aleutus skins and scales in concentrated HCl and crystallizing the solution.

Comparison of BioSea and other Formulations

Repeated experiments with BioSea 14 have proven this formula acceptable to the mussel embryos, but there is still need for considerable refinement. Table 2 compares the percent normal larvae of some commercial and artificial seawater formulations with real seawater and BioSea. It should be stated that all formulas of Table 2 produce some normal larvae when they contain Leslie NaCl and only abnormal larvae when purified NaCl is used.

When Leslie coarse hide salt is used as the source of NaCl, the percent normal larvae from the Lyman-Fleming and the Brujewicz formulas approximate those from real seawater and BioSea but embryo size and activity are less, and the general appearance of the larvae is poor. Herbst's formulation, though quite similar to BioSea, did not produce many normal veligers. The possibility exists, that the HCO_3^- was in the wrong proportion.

The commercial preparations--Instant Ocean, Rila Marine Mix and Magic Sea Salts--caused total mortality and/or complete disintegration of the eggs and embryos. It has been reported however, that these preparations are excellent for maintaining juvenile and adult marine animals. Tropic Marin, a German mixture, was the only commercial sea salt tested that produced normal larvae. Unfortunately, the formula is unavailable and apparently this product

Table 2. Comparison of percent normal larvae from some published and commercial formulas, seawater and BioSea.

♀	Yaquina Bay Salt Water	BioSea 14	Lyman & Fleming	Brujewicz	Herbst	Tropic Marin	Instant Ocean	Magic Sea Salt	Rila Maine Mix
1	52	60	57	56	5	--	0	0	0
2	87	84	85	78	5	--	0	0	0
3	49	59	59	51	5	--	0	0	0
4	82	87	--	--	--	55	0	0	0
5	88	85	--	--	--	41	0	0	0
6	90	87	--	--	--	48	0	0	0

is no longer offered for sale.

The small and mishapen veligers produced by the other formulas tested may have been caused by the trace elements or other superfluous compounds in the mixtures. This is possible even though some of the other formulas imitate the various ion concentrations of real seawater more closely than does BioSea. The comparison of the ion concentrations considered essential in BioSea with those of real seawater (Appendix D) are shown in Table 3. It can be seen that real seawater contains approximately 49.2% more magnesium, 39% more potassium, 20% more calcium, 2.7% more chlorine and 2.2% more sulfur than BioSea. Conversely, BioSea is richer in sodium by 8 percent. Wilbur and Yonge (1964) showed that the blood of adult mussels was greater in potassium and calcium, less in sulfur and equal in the sodium, magnesium and chlorine concentration of seawater. The blood of the larvae, could of course, be much different from that of the adult form. The fact that BioSea differs from real seawater chemically does not preclude the fact that embryos of M. edulis develop normally and thrive in this mixture.

Comparison of BioSea and Seawater

Larvae raised in BioSea for 52 days and fed Monochrysis sp. algae which was also grown in BioSea showed excellent growth and

in every way appeared normal. Figure 1 shows the mean sizes of 10 larvae at various times during the study. The veligers grew from a mean of 91 to 312 microns. Maximum growth was observed between the 11th and 25th day during which time their size increased by a mean of 114 microns. The first sign of foot development appeared after 40 days and the experiment was terminated when the larvae began to metamorphose and attach themselves to the sides of the aquaria with their bysuss threads.

Table 3. Differences in essential ion concentrations for the development of M. edulis between real seawater and BioSea at 34 ppt salinity.

Seawater	BioSea 14	Percent Difference	
gm/l	g/l		
Na^+ 10.60	11.52	8.0	greater in BioSea
Ca^{++} 0.40	0.32	20.0	Greater in real seawater
K^+ 0.38	0.23	39.0	
Mg^{++} 1.30	0.66	49.2	
Cl^- 19.00	18.48	2.7	
S 0.90	0.88	2.2	
HCO_3^- ?	0.128		
C 0.028 / 32.608	? / 32.218		

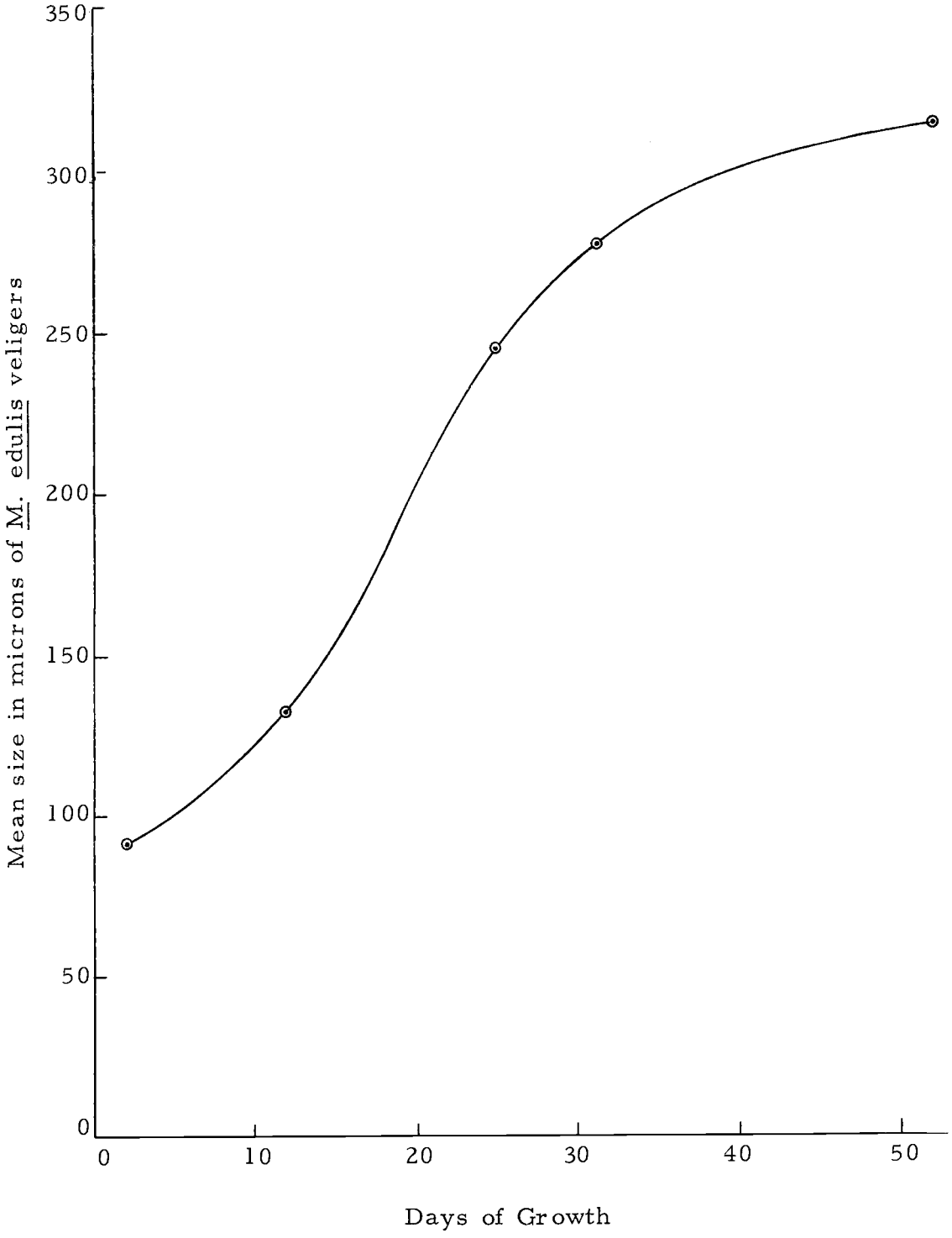


Figure 1. Growth of M. edulis larvae reared in BioSea for 52 days at 16°C and 25 ppt. salinity.

Figure 2 shows the percent normal larvae obtained in BioSea and plotted on the abcissa with the percent normal larvae in real seawater plotted on the ordinate for 60 tests. Points above the tranverse line favors the larvae in real seawater while points below the line favor the larvae in BioSea. Figure 2 substantiates the applicability of BioSea as an acceptable embryonic bioassay medium and shows that real seawater is slightly better for producing normal larvae than BioSea. It is apparent upon inspection of Figure 2 that eggs from different females produce varying percentages of normal larvae. This increases the problem of formulating a synthetic seawater because the relationship between egg quality and maturity to the amounts and kinds of chemical compounds necessary for its development are unknown. Nevertheless, the simplified formula of BioSea parallels real seawater with respect to the numbers of normal larvae produced.

The data for Figure 2 show the mean percent normal larvae in real seawater to be 78.8% and 77.2% in BioSea. Since there are differences in egg quality between females, most bioassay results are put on equivalent bases by considering all controls to be 100% and adjusting the test values accordingly (Dimick and Breese 1965). Relatively speaking then, the percent normal larvae in real seawater would be 100% and 98% in BioSea.

There are times when real seawater becomes toxic, due to

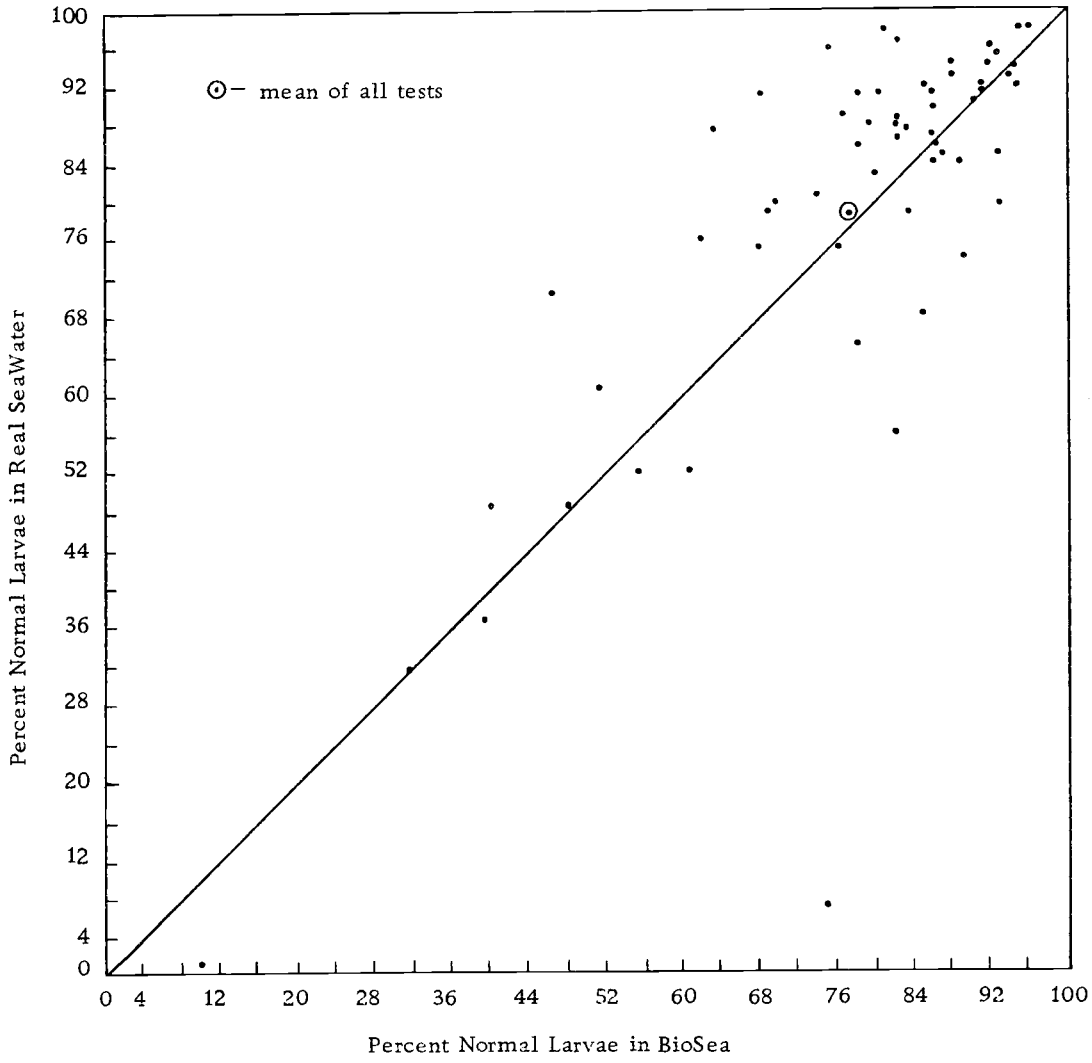


Figure 2. Comparison of percent normal larvae in BioSea and real seawater at salinity of 25 ppt.

algae decomposition and the subsequent release of metabolites or for other reasons. By using a tested synthetic seawater check, one could determine if a specific sample of seawater was toxic or if the low percentage of normal larvae observed was due to poor egg quality of the female. An example of poor quality seawater may be seen in Figure 2 where 75% of the larvae in BioSea developed normally, while in real seawater only 5% normal larvae developed.

Organic Requirements of *Mytilus edulis*: L Factor

Since the only formulas which produced shelled larvae contained the coarse NaCl, it is obvious that the key to successful shellfish development is associated with this impure compound. The data in columns 4 and 5 of Table 4 suggest that the essential unknown component is organic.

Notice that the numbers of normal larvae are reduced when the NaCl is kiln dried and are completely absent when the salt is burned according to Standard Methods (1960). It is possible that the burning of the salt recombines or in some way changes the inorganic impurities, but is clear that the associated organic molecules are destroyed. The essential and unknown organic material will hereafter be referred to as the L factor (Leslie coarse hide salt factor).

When natural seawater is evaporated, many compounds are precipitated and considerable quantities of the composite salts cannot

Table 4. Comparison of percent normal larvae in BioSea compounded with NaCl from various sources.

♀ *	Real Salt Water	BioSea 14 Leslie Coarse NaCl	Leslie Kiln Dried	Leslie Crude-Burned	Dried & Reconstituted Bay Salt	Salt Lake NaCl	Mortons Mined NaCl	Reagent NaCl	U.S.P. NaCl	Technical NaCl
1	91	75	52	0	0	few	few	0	0	0
2	97	91	54	0	0	few	few	0	0	0
3	96	85	27	0	0	few	few	0	0	0
4	90	82	--	0	0	few	few	0	0	0
5	85	84	--	2.7	0	few	few	0	0	0
6	88	79	--	0.7	0	few	few	0	0	0

* All tests run in duplicate.

be redissolved, unless acidified. Very few normal larvae are obtained when seawater is dried and reconstituted or from formulas made with crude NaCl from Great Salt Lake, Utah.

Reagent, U.S.P., or technical grades of NaCl will not produce normal larvae past the 6 hour trochophore stage. It was suggested that during the purification process, the NaCl might be contaminated with trace chemicals and therefore toxic to Mytilus. To test this hypothesis, seawater salinites were adjusted to 5, 10, 15, 20, and 25 ppt respectively by addition of distilled water. Then, the appropriate amount of coarse or reagent NaCl was added to bring all salinities to 25 ppt. This procedure was duplicated using reagent and coarse hide NaCl in BioSea 14. The percent normal larvae was then determined in each test vessel. Some of the observations are tabulated in Table 5. Tables 5a and 5b show that with 20% real seawater and sufficient coarse or reagent NaCl to make a salinity of 25 ppt, the percent normal larvae were equal to controls. But, no normal embryos were obtained with Leslie coarse hide salt by itself or with reagent NaCl by itself.

It may be concluded that reagent NaCl is non-toxic and that 20% real seawater contains enough of the other salts, trace elements and organic compounds for normal development of M. edulis, providing that the overall salinity is osmotically suitable to the embryo. In addition, Table 5c shows the great difference between the coarse

Table 5. Comparison of percent normal larvae using reagent and coarse NaCl mixtures in real seawater and BioSea.

5A. Salinity from seawater ppt	Coarse hide leslie NaCl Added Grams	Testing salinity ppt	Percent normal larvae
25	0	25	89
20	5	25	89
15	10	25	89
10	15	25	89
5	20	25	89
0	25	25	0

5B. Salinity from seawater ppt	Reagent NaCl Added Grams	Testing salinity	Percent normal larvae
25	0	25	86
20	5	25	87
15	10	25	89
10	15	25	87
5	20	25	86
0	25	25	0

5C. Reagent NaCl BioSea	Coarse NaCl BioSea	Testing salinity	Percent normal larvae
25	0	25	0
20	5	25	0
15	10	25	15
10	15	25	62
5	20	25	88
0	25	25	85

and reagent NaCl in terms of normal mussel development. Here, at least 80% of the coarse NaCl must be present before results equivalent to controls are possible. In comparison of the three tables, it becomes evident that a formula containing 80% coarse NaCl is about equivalent to a 20% seawater solution; or that seawater is 4 times richer in the L factor than BioSea.

A typical analysis provided for Leslie coarse NaCl shows that in 100 grams of the salt there is also 50 mg $MgSO_4$, 100 mg $MgCl_2$, 220 mg $CaSO_4$ and 20 mg of SiO_2 and R_2O_3 (contents not specified). It is felt that the L factor is contained in the 20 mg of organic/silicon complex, since the biological value of the salt is destroyed upon burning. The active L factor however, is undoubtedly much less than 20 milligrams. The possibility of chemically separating and analyzing this minute quantity of organic material from such an intense concentration of NaCl is extremely remote. At best, only some amino acids and molecular fragments of other organic compounds could be identified in light of today's analytical equipment and procedures (personal communication, Dr. Becker, Biochemist, Oregon State University).

Organic Compounds of Value to *Mytilus edulis* Embryos

With the foregoing in mind, the only recourse for identifying the L factor is the trial and error method of adding likely organic

compounds to the basic formulas using both reagent and coarse NaCl mixtures. Approximately 100 compounds, organic, inorganic and combinations thereof have been tested (Appendix C, E, and F). Of these, only 5 have consistently favored the embryonic development of M. edulis. Furthermore the biological usefulness of the 5 compounds have been substantiated by other investigators using various organisms. The compounds are DL lysine HCl, DL aspartic acid, alkaline phosphatase, carbonic anhydrase, and the free base purine (molecular weight 120) produced by the Nutritional Biochemical Company. The other organic compounds tested with four exceptions, are listed in Appendix E and are considered to be either non-effective or detrimental to the developing mussel embryos. The exceptions are pyrimidine, threonine, L cystine and L proline, which were helpful in initiating some type of starting shell material in larvae which were developing in BioSea made with reagent NaCl. However, no shelled larvae materialized in the completely reagent salt mixtures whether or not organic materials were added.

Typical effects of the organic compounds which have aided the developing mussel larvae are shown in Table 6. The enzymes, alkaline phosphatase and carbonic anhydrase, significantly increased the number of normal larvae, but only slightly increased their size. Lysine, aspartic acid and free base purine also increased the number of normal larvae, however an overall size decrease of about 1

Table 6. Organic compounds which aid the development of M. edulis in BioSea 14.

	% Normal larvae	Mean size of ten larvae in microns	Activity	General Appearance
Real Seawater Control	92.7	102.4	Very Active	Excellent
BioSea 14 Control	87.7	100.9	Moderate	Good
BS14 + DL Lysine 0.00033 mg/100 ml	92.7	99.3	Moderate	Good
BS14 + DL Aspartic Acid 0.033 mg/100 ml	93.4	100.8	Very Active	Excellent
BS14 + Alkaline Phosphatase a 0.033 mg/100 ml	94.0	101.5	Very Active	Excellent
BS14 + Purine (free base) a 0.033 mg/100 ml	93.4	99.8	Very Active	Excellent
BS14 + Carbonic Anhydrase a 0.033 mg/100 ml	94.7	100.1	Moderate	Excellent
BS14 + Threonine a 0.033 mg/100 ml	80.0	107.0	Very Active	Excellent
BS14 + Alkaline Phosphatase Purine (free base) DL Aspartic Acid DL Lysine HCl a 0.033 mg/100 ml	95.4	100.0	Very Active	Excellent
BS14 + Carbonic Anhydrase, Alkaline, Phosphatase, Purine (free base) DL Aspartic Acid DL Lysine HCl a 0.033 mg/100 ml	96.0	101.0	Very Active	Excellent
Pyrimidine L cystine L Proline	Helpful in reagent NaCl mixture only.			

micron occurred. When threonine alone was added, a mean size increase of 6 microns was observed, but this was accompanied by a reduction of 8% in normal larvae. Combinations of the enzymes, purine, lysine and aspartic acid resulted in an increase of 10% normal larvae, without changing their size. When these organic compounds were present, the activity and general appearance of the larvae were comparable to the real seawater controls.

BioSea with Organic Additives

The best synthetic seawater formulation for M. edulis embryos is shown in Table 7. It provides for well-shelled veligers, with good activity and size and there is no detectable chemical interference with toxicants such as complex Kraft pulp mill effluents. The formula contains the essential inorganic components plus the intrinsic unknowns associated with the Leslie coarse NaCl. It should be stated that the chemicals should be dissolved independently in the order in which they appear in the formula. Calcium chloride should always be added last and never mixed in the dry state with $MgSO_4$, because the calcium will displace the magnesium and form the relatively insoluble $CaSO_4$. This will result in few, if any, normal larvae.

Table 7. Suggested BioSea formula for Mytilus edulis embryos with organic additives.

I. Essential compounds for normal development.

Compound		Chemical quality
NaCl	33.0 gm	Leslie coarse hide salt
$NaHCO_3$	0.2 "	U.S.P.
KCl	0.5 "	U.S.P.
Anhy. $MgSO_4$	3.75 "	Reagent
$CaCl_2$	1.0 "	Reagent
	38.45 grams	

The salts are added in order to 1462 mls of glass distilled water to obtain a salinity of 25 ppt. Each salt should be dissolved before the next is added.

II. Organic compounds which consistently aid in normal development.

Alkaline phosphatase	0.33 mg/l
Carbonic anhydrase	0.33 "
Purine (free base)	0.33 "
DL Aspartic acid	0.33 "
DL Lysine HCl	0.00033 mg/l

III. Organic compounds which frequently aid in calcification and increase larval size. Results are inconclusive however, as detrimental effects have also been observed.

Pyrimidine	0.33 mg/l
L Cystine	0.033 "
L Proline	0.033 "
DL Threonine	0.0033 mg/l

Anomalies Among Mytilus edulis Embryos

It is important to note that anhydrous $MgSO_4$ and KCl appear to be compounds of critical concentrations as they cannot be altered more or less than 0.3 gm/liter without adversely affecting the quantity of normal larvae. Increasing either the magnesium or potassium concentration results in exploded and black spot anomalies. Drawings of observed anomaly types may be seen in Figure 3. The $NaHCO_3$ is also important because it buffers the water around pH 7.9 to 8.2, the pH of natural seawater, and this pH is apparently acceptable to the mussel. Hollow anomalies dominate when $NaHCO_3$ is lacking and with excess of HCO_3^- the embryos disintegrate. If $CaCl_2$ is lacking or the shell gland is injured, a black spot or earmuffed larvae is visible. This type is frequently seen in reagent NaCl formulations and Kraft waste bioassays.

The organic additives in BioSea are considered optional, as the embryo will develop a shell without them. However, the size, activity and general appearance of the larvae is considerably improved when the organic compounds are included. These compounds have definite biological functions. The enzyme, alkaline phosphatase, is found in the middle fold of the mantle tissue and is associated in some manner with molluscan calcification. Another enzyme, carbonic anhydrase, controls the rate at which CO_2 combines with water

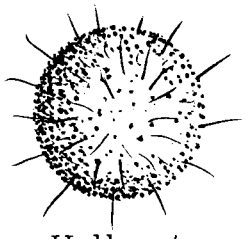
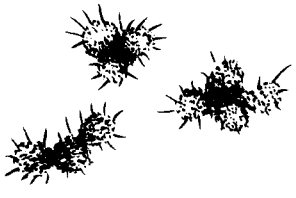
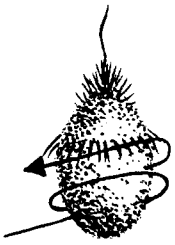
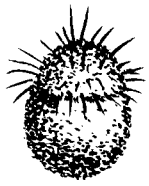
General appearance	Possible cause
 1. Hollow type	Incorrect concentration of HCO_3^-. Other unknown.
 2. Ear muffed	Lack of CO_2. Lack of organic unknown. Damage to shell gland. Inhibition of shell enzymes. Incorrect concentration $CaCl_2$.
 3. Black spot	Incorrect concentration $MgSO_4$. Malfunction of shell gland. Inhibition of shell enzymes.
 4. Exploded but swimming	Incorrect concentration $MgSO_4$. Improper osmotic balance.

Figure 3. Types of Abnormal M. edulis Embryos.



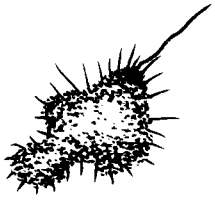
5. Levorotary spin

Cause unknown, this is a rare anomalie. Normal trochophore spin is dextrorotary.



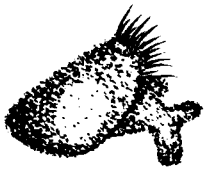
6. Lacking or reduced apical flagella

Unknown.



7. Malformed trochophore

Improper osmotic balance. Other unknown.



8. Viscera extruded from shell

Unknown.

9. Normal appearing trochophore ceases development

Lack of CO_2. Malfunction of shell gland and shell enzymes. Other unknown.

Figure 3. (continued)

in an organism and has been shown to be closely related with the calcifying process (Freeman and Wilbur, 1948).

The amino acids, threonine and lysine are considered to be essential for growth and aspartic acid is considered a growth stimulator but is nonessential. Aspartic acid is found in large quantities in seawater and provides a raw material from which other organic compounds may be derived.

The purines and pyrimidines are essential for synthesis of nucleic acids and the embryos appear to profit by their presence in the formula.

Outlook for BioSea

Since there are other organic compounds and especially enzymes which appear to be closely associated with molluscan calcification it is necessary to continue this research before a completely synthetic seawater can be mixed with reagents from the shelf. Until that time however, when the L factor is identified, Leslie's coarse NaCl can suffice for the essential unknowns.

Currently, BioSea is being used on a routine basis at the Oregon State University Marine Science Center, Newport, Oregon to monitor the quality of the bay water used there. It is also regularly added to raise salinities and to fortify liquid toxicants where high concentrations and salinities are not possible by any other means.

Present graduate research involving the treatment and degradation of Kraft pulp mill effluents, frequently uses BioSea in this manner.

Data on assays of Kraft mill waste with seawater and with BioSea are given in Table 8. No biological or chemical interference was apparent when Kraft mill wastes bioassays were run with BioSea. The numbers of normal larvae are essentially the same in the artificial and real seawater and the mean lethal tolerance limits (TLm) are essentially identical. There is considerable variability among females, but ova from a given female yield similar data in Kraft bioassays with seawater or BioSea.

Embryonic Anomalies

Studying the abnormal larvae and understanding the mechanisms responsible for the abnormality may shed information on molluscan calcification and possibly suggest new methods of treating industrial wastes to reduce their toxicity. For instance, exploded and black spot anomalies (Figure 3) may be observed in Kraft pulp mill effluents. Similar anomalies can be caused by shifting the $MgSO_4$ concentration in BioSea. This suggests that the complex organic Kraft wastes, when sewered into the ocean, may in some way interfere with magnesium metabolism in Mytilus embryos.

Further experiments indicate that when $MgSO_4$ is added to Kraft effluent a precipitate readily forms. Bioassays of the mother

Table 8. Comparison of Kraft Pulp Mill bioassays results in BioSea and real seawater at 25 ppt salinity.

Real S. W.	% N. larvae	BioSea	% N. larvae
Control	93.4	Control	92.0
1% Kraft	90.0	1% Kraft	82.7
2 "	77.4	2 "	76.0
3 "	32.4	3 "	34.0
4 "	0.0	4 "	0.0
	TLm = 2.7		TLm = 2.7
Control	80.0	Control	82.7
1% Kraft	74.7	1% Kraft	73.4
2 "	58.0	2 "	59.4
3 "	33.0	3 "	36.7
4 "	0.0	4 "	0.0
	TLm = 2.7		TLm = 2.7

liquor after $MgSO_4$ precipitation shows that a 50% or greater reduction in toxicity has occurred. When other divalent cations are used to precipitate Kraft wastes, the toxicity of the mother liquors remains unchanged.

In laboratory experiments with BioSea and seawater, many embryos develop to the ciliated and swimming trochophore stage. When this level is reached, the organism rises to the surface and makes contact with the air-water interface. At the interface the trochophore is suspended by its apical flagellum in the meniscus of the seawater. This migration to the surface is repeated many times and lasts a few seconds to several minutes. Calcification will not occur unless an air-water interface is available, even though the oxygen content may be 6 mg/liter or more. Development of Mytilus in a stoppered bottle filled to the top, with seawater or BioSea, and having no air-water interface, will proceed only to the trochophore stage. Many of the resulting anomalies when an air-water interface is lacking, are of the ear muff and black spot type (Breese and Courtright, unpublished). The roles of metabolic carbon dioxide and carbonic anhydrase in shell formulation are well documented (Jodrey, 1953) (Freeman and Wilbur, 1948), and their parts in initiating and continuing calcification of Mytilus trochophores should be investigated.

Future Studies

BioSea represents a standard reproducible medium for developing Mytilus embryos. In general, however, natural seawater is usually the preferable medium for the growth and development of Mytilus edulis. But BioSea does not vary, while seawater, particularly in estuaries with commercial and urban effluents, can vary greatly. BioSea represents a standard for measuring the biological value of seawaters from different places and different times with respect to embryos of Mytilus. It is hoped that other investigators will explore the potential of BioSea as a standard marine bioassay water with other organisms and other waste materials. Further research is needed to identify the L factor (essential factor in Leslies coarse hide salt) responsible for molluscan calcification.

SUMMARY

1. Commercial preparations and published formulations using reagent chemicals for seawater substitutes were not biologically acceptable to Mytilus edulis embryos, in that calcification did not occur.

2. When Leslie coarse hide salt was used as the source of NaCl for literature formulations, most formulas provided some success in the development, growth and calcification of mussel embryos.

3. By varying concentrations of several anions and cations, and using Leslie coarse hide salt, a new formula (BioSea) was developed which would allow normal growth and development to the shelled veliger stage.

4. Essential inorganic compounds for M. edulis development are NaCl, $NaHCO_3$, KCl, anhydrous $MgSO_4$ and $CaCl_2$.

5. The Leslie coarse hide salt (NaCl) used in BioSea contains an essential compound or compounds (L factor), thought to be organic, necessary for calcification of mussel larvae. This factor is destroyed upon burning of the salt.

6. Seawater contains 4 times as much of the essential L factor as BioSea. A solution of 20% real seawater, with the salinity adjusted to 25 ppt using reagent NaCl will provide all necessary compounds for normal development of M. edulis.

7. Growth and development of Mytilus was improved by adding

alkaline phosphatase, carbonic anhydrase, purine (free base), DL lysine and DL aspartic acid to BioSea.

8. Abnormal larvae could often be related to specific substances or specific procedures. Nine abnormalities of frequent occurrence were described. These included hollow embryos, ear muff, black spot and exploded types, levorotory spin, malformed trochophores, trochophores without flagella and larvae with extrusion of viscers.

9. Results of mussel bioassays with Kraft mill effluents in BioSea were equivalent to those using natural seawater. It is suggested that BioSea be used as standard media for bioassays of marine waters. Additional research is strongly recommended towards identifying the L factor (Leslie coarse hide salt factor) which is necessary for calcification of shellfish larvae.

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APPENDICES

APPENDIX A

I. COMMERCIAL PREPARATIONS

A. INSTANT OCEAN Synthetic Sea Salts

Formula for One Hundred Gallons of Artificial Sea Water

COMPOUND	AMOUNT
NaCl	23.0 lb.
$MgCl_2$	4.5 lb.
$MgSO_4$	5.75 lb.
$CaCl_2$	1.15 lb.
KCl	9.8 lb.
$NaHCO_3$	2.8 oz.
$SrCl_2 \cdot 6H_2O$	7.5 G.
$MnSO_4 \cdot H_2O$	1.5 G.
$Na_2HPO_4 \cdot 7H_2O$	1.5 G.
LiCl	.375 G.
$Na_2M_oO_4 \cdot 2H_2O$	.375 G.
$Na_2S_2O_3 \cdot 5H_2O$	.375 G.

To above mixture add 160 ml of the trace element stock solution.

Trace element stock solution:

Dissolve in 4 liters distilled water, (* requires heat) then mix.

* $Ca(C_6H_{11}O_7)_2$	6.25 G.
KI	.9 G.
KB_r	9.52 oz.
* $Al_2(SO_4)_3$	4.5 G.
* $CoSO_4$	.5 G.
RbCl	1.5 G.
$CuSO_4 \cdot 5H_2O$	.1 G.
$SnSO_4 \cdot 7H_2O$	.96 G.

Add salt mixture with 160 ml of trace element stock solution to 100 Gallon tap water.

APPENDIX A

B. RILA MARINE MIX

RILA PRODUCTS
P. O. BOX 114
TEANECK, N. J.

In 100 mls. of solution

NaCl	2640.0000 mg	Co (as SO_4)	00.0070 mg
$MgCl_2 \cdot 6H_2O$	508.0000 mg	Fe (as SO_4)	00.0100 mg
$MgSO_4$	605.0000 mg	Zn (as SO_4)	00.0200 mg
KCl	64.0000 mg	Mn (as SO_4)	00.0700 mg
Ca (as Cl^-)	106.0000 mg	Pb (as SO_4)	00.0010 mg
Ca (as SO_4)	30.0000 mg	Cu (as SO_4)	00.0001 mg
Sr (as Cl^-)	0.3800 mg	KI	2.0000 mg
Al (as Cl^-)	0.0030 mg	KBr	2.2000 mg
Rb (as Cl^-)	0.0006 mg	KNO_3	10.0000 mg
Li (as Cl^-)	0.0006 mg	K_2HPO_4	1.0000 mg
Mo (as MaMoO)	0.0020 mg	$Na_2SiO_3 \cdot 9H_2O$	10.0000 mg

C. MAGIC SEA SALTS

(Formula Unknown)

D. TROPIC MARIN

(Formula Unknown)

APPENDIX A

II. FORMULAS IN LITERATURE

E. McClendon, et al. 1917 (Sverdrup, Johnson and Fleming, 1946)

	gms.
NaCl	26.726
KCl	0.721
$MgSO_4$	3.248
$MgCl_2$	2.260
$CaCl_2$	1.153
NaBr	0.058
$NaHCO_3$	0.198
Na_2SiO_3	0.0024
$Na_2Si_4O_9$	0.0015
H_3PO_4	0.0002
H_3BO_3	0.058
Al_2Cl_6	0.013
NH_3	0.002
$LiNO_3$	0.0013
	34.4406

add water to 1000 grams

F. Brujewicz, 1931 (Sverdrup, Johnson and Fleming, 1946)

NaCl	26.5 gms/l
KCl	0.7
$CaCl_2$	1.1
$MgCl_2$	2.5
$MgSO_4$	3.3
$NaHCO_3$	0.2
NaBr	0.08
	34.38

add water to 1 liter

II. FORMULAS IN LITERATURE
(Continued)

G. Lymans and Fleming, 1940 (Sverdrup, Johnson and Fleming, 1946)

NaCl	23.476 gms/Kg
$MgCl_2$	4.981
Na_2SO_4	3.917
$CaCl_2$	1.102
KCl	0.664
$NaHCO_3$	0.192
KBr	0.096
H_3BO_3	0.026
$SrCl_2$	0.024
NaF	0.003
	34.491

add water to 1000 grams

H. Herbst (Lutz, F. E. et al., 1937)

NaCl	30.0 gms/l
KCl	0.8
$MgSO_4$	6.6
$CaCl_2$	1.3
$NaHCO_3$	0.005
	38.705

add water to desired salinity

II. FORMULAS IN LITERATURE
(Continued)

I. Von Flock's (Lutz, F. E. et al., 1937)

NaCl	28.15 gms/l
$CaSO_4 . 2H_2O$	1.72
$MgSO_4 . 7H_2O$	3.20
$MgCl_2 . 6H_2O$	8.50
KCl	0.80
$MgBr_2$	0.10
	32.30

add 1 liter of water

J. Schmalz (Lutz, F. E. et al., 1937)

NaCl	28.15 gms/l
KCl	0.67
$MgCl_2 . 6H_2O$	5.51
$MgSO_4 . 7H_2O$	6.92
$CaCl_2 . H_2O$	1.45
	32.70

add 1 liter of water

APPENDIX B

I. Sodium Chloride tested from various sources.
   A. Only abnormal larvae occurred.
      1. Reagent grade
      2. U.S.P.
      3. Technical
      4. Leslie water softener salt
      5. Leslie coarse hide salt (burned)

   B. Only poorly shelled larvae occurred.
      1. Salt Lake, Utah, Salt
      2. Sea salt (by air evaporation)
      3. Sea salt (by boiling to dryness)
      4. Leslie kiln dried salt
      5. Morton mill run salt

   C. Normal, well developed veligers occurred.
      1. Leslie coarse hide salt, solar evaporated

II. Water tested from various sources.
   A. Suitable for use as make-up water in BioSea formulations.
      1. Glass distilled
      2. Rain water
      3. Spring water (near Yaquina Bay, laboratory)
      4. Oak Creek laboratory water, O.S.U. Corvallis, Oregon

   B. Only poorly shelled larvae occurred.
      1. Charcoal filtered, O.S.U. Marine Science Lab., Water
      2. Salt Lake, Utah, water salinity 270 ppt. diluted with glass distilled water to testing salinity, and other BioSea salts added.

   C. Only abnormal larvae occurred.
      1. City of Newport, Oregon tap water
      2. Water distilled from a copper tank.

APPENDIX C

The following compounds were either non-effective or detrimental to developing M. edulis embryos when added to BioSea 14. Concentrations approximated those in real seawater.

I. Salts not effecting calcification of M. edulis in BioSea.

Compound	Concentration in mg/liter
1. KNO_3	100
2. K_2HPO_4	10
3. KBr	20
4. NaBr	22
5. Na_2SO_4	3900
6. $NaNO_3$	10
7. Ca Glycerophosphate	10
8. V_2O_5 as vanadium	0.002
9. MgSiF	0.02
10. $Al_2(SO_4)_3$	0.03
11. RbCl	0.006
12. $MnSO_4$	0.7

APPENDIX C
(Continued)

II. Salts detrimental to developing M. edulis embryos.

Compound	Concentration in mg/l
1. KI	20
2. $FeCl_3$	1
3. NaN_3	0.1
4. Na Molyodate	.02
5. Ca Lactate	10
6. Ca Gluconate	10
7. H_3BO_3	58
8. H_3PO_4	0.2
9. $CuSO_4$	0.003
10. LiCl	0.006
11. $ZnCl_2$	0.2
12. $CoCl_2$	0.08
13. NaF	3
14. $SrCl_2$	7.5
15. $Na_2SiO_3 \cdot 9H_2O$	100

APPENDIX D

KNOWN ABUNDANCES IN SEA WATER

Element	mg./l.	g/l.
Chlorine	19,000	19.
Sodium	10,600	10.6
Magnesium	1,300	1.3
Sulfur	900	0.9
Calcium	400	0.4
Potassium	380	0.38
Bromine	65	0.065
Carbon	28	0.028
Oxygen	8	0.008
Strontium	8	0.008
Boron	4.8	.0048
Silicon	3.0	.003
Fluorine	1.3	.0013
Nitrogen	0.8	.0008
Argon	0.6	.0006
Lithium	0.2	.0002
Rubidium	0.12	.00012
Phosphorus	0.07	.00007
Iodine	0.05	.00005
Barium	0.03	.00003
Indium	0.02	.00002
Aluminum	0.01	.00001
Iron	0.01	.00001
Zinc	0.01	.00001
Molybdenum	0.01	.00001
Selenium	0.004	.000004
Copper	0.003	.000003
Arsenic	0.003	.000003
Tin	0.003	.000003
Lead	0.003	.000003
Uranium	0.003	.000003
Vanadium	0.002	.000002
Manganese	0.002	.000002
Titanium	0.001	.000001
Thorium	0.007	.0000007
Cobalt	0.005	.0000005

APPENDIX E

The following compounds were tested with developing M. edulis embryos, in BioSea 14.
Concentrations used were 0.033, 0.00033, and 0.000033 mg/100 ml respectively.

I. Non-effective towards calcification of M. edulis embryos.

1. L Arginine HCl
2. L Asparagine
3. L Cysteine HCl
4. L Glutamic Acid
5. L Histidine
6. DL Alpha Alanine
7. DL Isoleucine
8. DL Leucine
9. DL Methionine
10. DL Norleucine
11. DL Serine
12. DL Tyrosine
13. Valine
14. Beta Alanine
15. 7-Dehydro Cholesterol
16. Ribonucleic Acid
17. Desoxy Ribonucleic Acid, Highly Polymerized
18. Dl Tryptophane
19. Glycyl Glycine
20. Urea
21. Yeast
22. Citric Acid
23. Periostracum Sulfate (Fields 1922)

II. Detrimental towards calcification of M. edulis embryos.

1. DL Phenylalanine
2. Aminoacetic Acid (Glycine)
3. Adenosine Triphosphate Crystalline (Disodium)
4. L Aspartic Decarboxylase
5. Dimethyl Sulfoxide
6. Ethylene Diamine Tetraacetic Acid

APPENDIX E (Continued)

7. Calciferol
8. Testoserone
9. Barnacle extract
10. Human urine
11. Ringers Solutions
12. Ringers Culture Media
13. Adenylic Acid (Muscle)
14. Trishydroxymethylamino Methane

III. Possible effectiveness towards calcification of M. edulis embryos.

1. DL Methionine
2. Dl Norvaline
3. Yeast Extract Powder
4. Casein (Hammerstein)
5. Polyopterin

Organic Compounds Used in Combinations

I. Non-effective towards calcification of M. edulis embryos.

1. L Proline, L Arginine HCl, L Histidine HCl
   L Cysteine HCl, L Cystine, L Asparagine
   L Glutamic Acid, DL Aspartic Acid
   DL Threonine, DL Tryosine, DL Valine

2. DL Lysine HCl, DL Aspartic Acid
   Casein (Hammerstein) L Proline, L Arginine HCl
   L Histidine HCl, L Cysteine HCl, Cystine
   L Asparagine, L Glutamic Acid

3. L Arginine HCl, L Asparagine, L Cysteine HCl,
   L Cystine, L Glutamic Acid, L Histidine, L Proline

4. Beta Alanine, DL Norvaline
   DL Lysine, HCl, L Histidine HCl

APPENDIX E (Continued)

5. L Arginine HCl, DL Serine, L Cysteine HCl
   L Cystine, DL Tryosine, DL Valine
   L Proline, L Histidine HCl, L Glutamic Acid
   L Asparagine

II. Detrimental towards calcification of M. edulis embryos.

1. 7-Dehydro Cholestrol
   Adenosine Triphosphate (Disodium)
   L Aspartic Acid

2. 7-Dehydro Cholestrol, Adenosine Triphosphate
   L Aspartic Decarboxylase
   Desoxy Ribonucleic Acid (Highly polymericed)

3. 7-Dehydro Cholestrol, L Aspartic Decarboxylase
   Desoxy Ribonucleic Acid (H. P.)

4. 7-Dehydro Cholesterol, Riboncleic Acid
   Adensoine Triposphate (Disodium)
   L Aspartic Decarboxylase
   Desoxy Ribonucleic Acid (H. P.)
   Di-Methyl Sulfoxide-100 drops of stock (5 drops/300 ml H_2O)

III. Possible effectiveness towards calcification of M. edulis embryos.

1. L Proline, L Arginine HCl, L Histidine HCl
   L Cysteine HCl, L Cystine, L Asparagine
   L Glutamic Acid, DL Aspartic Acid
   DL Theonine, DL Tyrosine, DL Valine
   Pyrimidine, Alkaline Phosphatase

2. L Proline, Alkaline Phosphatase, Purine (free base)
   DL Aspartic Acid, DL Lysine HCl

3. DL Aspartic Acid, Casein (Hammerstein)
   Purine (free base)

APPENDIX E (Continued)

4. Purine (free base), casein (Hammerstein)
   Alkaline Phosphatase, DL Aspartic Acid
   DL Lysine HCl

5. Alkaline Phosphatase
   Purine (free base), L Proline, L Arginine HCl
   L Histidine HCl, L Cysteine, L Cystine
   L Asparagine HCl, L Glutamic Acid
   (5 ml of each mixed together from stock)

6. Pyrimidine, Alkaline Phosphatase
   L Proline, L Cystine

7. Beta Alanine, Pyrimidine, Alkaline Phosphatase
   L Cystine*, L Proline, L Arginine HCl
   L Histidine, L Cysteine, L Asparagine
   L Glutamic Acid---all 22 amino acids

IV. Effective towards calcification of M. edulis embroys.

1. Pyrimidine, Alkaline Phosphatase, L Cystine*
   L Proline, L Arginine HCl, L Histidine, L Cysteine
   L Asparagine, L Glutamic Acid
   DL Aspartic Acid, DL Threonine

* Not all material dissolved.

APPENDIX F

LESLIE SALT CO.
SAN FRANCISCO, CALIFORNIA

TYPICAL CHEMICAL ANALYSES OF UNTREATED COARSE HIDE SALT

		Received	Dry
H_2O at 500° F	Water	0.09%	0.00%
$CaSO_4$	Calcium Sulphate	0.17%	0.17%
$MgCl_2$	Magnesium Chloride	0.04%	0.04%
$MgSO_4$	Magnesium Sulphate	0.01%	0.01%
SiO_2&R_2O_3	Insolubles	0.01%	0.01%
NaCl	Sodium Chloride	99.68%	99.77%
		100.00%	100.00%