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AN EVALUATION OF THE TOXICITY AND SUBLETHAL EFFECTS OF ATRAZINE ON THE PHYSIOLOGY AND GROWTH PHASES OF THE AQUATIC MACROPHYTE VALLISNERIA AMERICANA L.

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AN EVALUATION OF THE TOXICITY AND SUBLETHAL EFFECTS OF ATRAZINE

ON THE PHYSIOLOGY AND GROWTH PHASES OF THE AQUATIC

MACROPHYTE VALLISNERIA AMERICANA L.

by

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Washington, D.C. 20016

DEDICATION

To Julie and Kafi

AN EVALUATION OF THE TOXICITY AND SUBLETHAL EFFECTS OF ATRAZINE ON THE PHYSIOLOGY AND GROWTH PHASES OF THE AQUATIC MACROPHYTE VALLISNERIA AMERICANA L.

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ABSTRACT

Sublethal effects of an herbicide on the life cycle of a submersed aquatic macrophyte were investigated. A unique microcosm was designed to accommodate long-term culturing of submersed macrophytes in the laboratory and to provide a medium for testing of toxic substances. The Gilson Differential Respirometer was modified for short-term toxicity testing. A computer program was written for the reduction of data obtained from the Respirometer (appendix).

Atrazine was utilized to assess the sublethal effects of a commonly used herbicide on a submersed aquatic plant, Vallisneria americana, one species which has experienced decline in distribution in the Chesapeake Bay.

The results of this research indicate that all phenophases of V. americana can be duplicated in these microcosms. V. americana exhibited a significant attrition in the life-cycle completion when exposed to sublethal concentrations of Atrazine. Every stage in the life cycle of V. americana was affected.

Leaf growth and whole plant biomass were significantly affected at all times during the growing season at concentrations above 8 ppb.

Sexual reproduction was affected by either a lack of flower production (female) or deterioration of the flower (male) above 16 ppb.

The ability of V. americana to over-winter as underground tubers was also significantly reduced at 4 ppb and above. Tuber number was reduced by 60% and tuber biomass (stored food) was reduced by 27% at 16 ppb.

Other research has documented ephemeral pulses of these sub-lethal concentrations of Atrazine in the headwaters of tributaries to the Chesapeake Bay, where V. americana commonly occurs. The occurrence of this herbicide in these tributaries in the author's estimation is largely due to misuse of this herbicide and farming practices which lend themselves to soil erosion into nearby bodies of water.

The results of this research indicate that Atrazine is as important as any other factor in contributing to the decline of V. americana.

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INTRODUCTION

Application of herbicides became an attractive means of mechanizing crop production in post World War II years, allowing more efficient use of personnel, and avoiding increased labor costs from hand-weeding. In fact, herbicides became the most widely used pesticide in agriculture. Their use in North America alone exceeded the combined totals of insecticides and fungicides (McEwen & Stephenson, 1979). A $1-$10 return per $1.00 investment in herbicides was quickly realized. Foremost in pre- and post-emergent control of weeds were the triazines, developed by J. R. Geigy Ltd. in Switzerland. Atrazine, which was released in 1957 by this company, had greater predictability for preemergence weed control and greater effectiveness for post-emergent weed control than Simazine, its predecessor.

During the 1970s, Atrazine led all other herbicides in volume used in the midwest cornbelt, where 83% of the corn crop is treated. Corn fields possess a variety of weeds which necessitate a broad spectrum herbicide such as Atrazine. Von Rumker et al. (1975) reported the annual use of 100 million lbs. of Atrazine.

Increasingly large amounts of Atrazine are being applied to the croplands in Maryland and Virginia surrounding the Chesapeake Bay (Stevenson, 1978). The use of Atrazine as a weed control accounts for the predominant reason why corn yields have dramatically increased in the last fifteen years. However, while making a major impact on total

bushels of corn harvested, the use of this herbicide is not without pitfalls.

Atrazine is a persistent herbicide, especially during dry years, and is carried over until the next season. Sensitive crops, such as soybeans, cannot be planted in the same fields. This leads to repeated plantings of corn in the same field year after year. Also, weed species become tolerant to Atrazine and eventually reduce the yield. The situation was demonstrated in a study by Radosevich (1973), in which he found tolerant biotypes of weeds which become dominant, and appeared in fields where corn treated with Atrazine had been repeatedly planted season after season.

The main environmental concern, due to the persistence and translocation of Atrazine, is its potential toxicity to non-target plants. This toxicity can occur to submersed aquatic vegetation as well as to terrestrial plants. Because it was strongly suspected that the increase of herbicides in the water column and adsorbed onto sediment was directly correlated with the abrupt decline of submersed vegetation in recent years, many monitoring programs, especially in the Chesapeake Bay region have been established (Correll et al., 1977; Norton & Ellis, 1976; Stevenson et al., 1978, 1980).

Since these studies have rarely isolated concentrations of herbicides, including Atrazine, above 40 ppb, many scientists discount this as a possible hazard to submersed vegetation. However, long-term effects by isolated ephemeral pulses of herbicides occurring at critical points in the life cycle of ecologically important submersed grasses, should not be underestimated. Sublethal stresses from these pulses, occurring at critical points in the life cycle, may have major impacts

on growth and reproduction, from which these grass beds may never recover.

Toxic effects and sublethal stresses can be studied from the cellular to the ecosystem level. This research complemented other research (Forney & Davis, 1981) associated with potential toxic effects of Atrazine on submersed vegetation, and explored areas that heretofore have remained untouched.

The approach taken for this study initially involved an extensive literature search collating numerous reports, articles, and papers into a single document on the chemical in question. Secondly, the life history of the chosen plant was closely followed in the field over two entire growing seasons and then simulated in laboratory microcosms.

The overall goal of this research was to examine if sublethal doses of one herbicide, Atrazine, posed a threat to an ecologically important submersed plant such as Vallisneria americana L., and whether these stresses had adverse effects on growth, reproduction, and photosynthesis.

CHAPTER I

SUBMERSED AQUATIC VEGETATION

Value to Aquatic Ecosystems

The importance of SAV to the Chesapeake Bay becomes evident when it is realized that these plants provide shelter, food, and breeding habitats for fish, shellfish, waterfowl, epiphytes, and many other aquatic organisms. Waterfowl are especially dependent on the seeds and new shoots produced by these plants. If densities of SAV become low, these birds will often seek new feeding ground, in search of a more plentiful crop.

Beds of SAV provide shelter for juvenile fish and crabs. This is especially important for the economically profitable blue crab in the Chesapeake Bay, since it is very vulnerable to predators during molting (because of the soft shell and sluggish activity). Submersed vegetation is a crucial link in an aquatic food web since these grasses are primary producers. Much of the above ground biomass annually decomposes to detrital food particles which in turn is available to benthic organisms, as well as other detritivores (Anderson, 1981).

Wave action is reduced where SAV grow, as well as tidal and wind driven currents in shallow areas. These grasses also reduce turbidity by trapping sediments from the water column. Erosion is decreased by this reduction in wave action and currents, since energy is dissipated before damage can be inflicted on the shoreline. Likewise, loss of

grassbeds increases turbidity, tends to bury existing vegetation, and reduces light, which, in turn, results in greater losses in vegetation.

SAV also plays a substantial role in nutrient buffering of a water column (Sculthorpe, 1967). These plants utilize dissolved nitrogen and phosphorus; making them unavailable for the algal growth, that often results in eutrophication in rivers that run into the Chesapeake Bay (Stevenson, 1978).

Decline of Submersed Vegetation in the Chesapeake Bay

Prior to the 1970s, SAV accounted for 40% of relative primary production. During the 1970s the submersed vegetation in the Bay experienced a decline. After the decline in the 1970s, SAV amounted to less than 10% of primary production (Anderson, 1981). This percentage can be compared to the estuarine sounds of North Carolina, which have a primary production of SAV of nearly 70%, with no indication of any decline.

In a survey by the U.S. Fish and Wildlife Services' Migratory Bird Habitat Research Laboratory, 28% of the stations were vegetated in 1971 compared to about 10% in 1978. Certain areas around the Bay were hardest hit: (1) the head of the Bay where the Susquehanna River enters. There, entire populations were eliminated; (2) the Patuxent River on the western shore was supported by eight species in the 1960s (Anderson, 1972), but only four were found in the 1970s; (3) the Calvert Cliff region had four species between 1930-70, but no grasses were found between 1971-76; (4) the South Marsh Island area near the Maryland-Virginia border was severely hit in 1976, while other areas were improving.

One of the most dramatic declines of SAV occurred in the Severn River on the western shore of the Bay (personal observation). In 1980-81, the entire river was surveyed and numerous species were mapped at various locations. In 1982, all but a few beds were entirely absent (personal observation). None of the former beds were found in 1983.

The overall decline in the Bay has been random. No pattern seems to exist, which raises the question whether SAV is experiencing a natural decline, or responding to man made impacts or both. It is important to bear in mind that the Bay recently has not been as resilient to storms as it was in the 1930s. It is entirely possible that a normal natural fluctuation has been compounded by excessive pollutants, including herbicides, from both point and nonpoint source variety. Currently, there is colonization of under 10% of all available habitats, defined as shoal area, 2 meters or less in depth, as determined by Anderson (1981) using a combination of aerial photography and ground survey by seaplane.

A number of environmental factors could have possibly caused the decline of SAVs in the Bay. For instance, Hurricane Agnes: this storm that hit the Bay in 1972 was comparable to the record storm in August 1933. Expected recovery after Agnes has not ocucrred, although there has been some localized recovery. There were some long-term effects such as in the Susquehanna Flats where large quantities of sediment buried the SAV. Since this and other areas have not recolonized twelve years later, the question remains as to why? There are suspicions that other factors are contributing to this lack of recolonization, including salinity, toxic chemicals, chlorine, eutrophication, and suspended sediments.

Salinity

Since most SAV in the Chesapeake Bay prefer fresh and brackish water (with the exception of Zostera), reduced salinities probably haven't been a significant factor in the decline. Likewise, excessive salinities as recently as 1981, did not appear to affect healthy beds of SAV. In one location on the Potomac River which was a normal maximum salinity in August of 6-7 ppt, saline water protruded farther upstream in 1981 than in 1980, 1982, or 1983. Salinities reached 10 ppt, and viable beds of SAV were noted (personal observation).

Heavy Metals and Petroleum Products

Although there is some indication that heavy metals and petroleum products negatively impact SAV, experimental data has been insufficient to determine the toxic effect of these substances or whether sufficient toxic levels exist in the Bay. In fact, one major area of industrial contamination is the Patapsco River and Baltimore Harbor. Here lush beds were recorded during the 1980-83 growing season (personal observation).

Chlorine

According to Stevenson (1979), 29.2 million pounds of chlorine entered Chesapeake Bay in 1973. An estimate of 3% of this amount could produce persistent by-products; namely, chlorinated hydrocarbons. Yearly increases in chlorine loading correlate with the 1971 decline to the present (Stevenson, 1979).

However, the major sources of chlorine contamination do not geographically coincide with the areas of major decline, and lush beds exist around or near these sources (personal observation).

Additionally, chlorine added to microcosm water in a concentration that is suggested for purification of drinking water, killed the above ground biomass of V. americana, but had no effect on rhizomes. Within a few weeks, new above ground biomass was produced (personal observation). Studies are lacking as to the effect of chlorinated hydrocarbons on the photosynthetic rate of SAV, therefore the role of these compounds in SAV decline cannot be discounted.

Excessive Nutrients

There is a large input of nutrients from untreated or partially treated municipal wastewater due to an increase in the effluents from growing metropolitan areas. Additionally, nonpoint sources of fertilizers contribute significantly to the nitrate and phosphate content of the water. It has been suggested that large algal blooms brought about by high nutrient input can cause a decline in grasses due to shading and light attenuation. The role of eutrophication on SAV decline is controversial, since extensive, healthy beds of various submersed plants have been observed in areas of obvious high nutrient input to adjacent waters (e.g., effluent from a trailer part, personal observation). Although epiphytic growth was quite heavy on leaves, no algal blooms were observed on the water surface and Vallisneria growth was lush with leaves exceeding one meter in length. Tuber production also was not impaired.

Additionally, during the 1950s, the Chesapeake Bay was affected by excessive growth of Myriophyllum and water chestnut (Trapa natans). These species successfully competed with other more desirable aquatic plants for light, nutrients, and space. Since 1960, the Upper Potomac

has experienced large algal blooms (Anacystis) on the water surface, brought about by high nitrogen and phosphate inputs. This has been a factor in the decline in grasses due to shading and light attenuation (Stevenson, 1978).

Suspended Sediments

Particulate matter in the water column causes a reduction of light penetration and also absorption of nutrients and gases by coating the leaves and stems. Unquestionably high sediment loads have contributed to decline of SAV beds in some tributaries of the upper Chesapeake Bay. V. americana, however, is very tolerant of low light levels. It maintains 25% of surface photosynthesis rate when light has been attenuated to ½% of I_0 (incident light impinging upon light surface). At one of the sites monitored in this research, turbidity was excessive, yet healthy beds were observed through 1980-83 growing seasons.

Herbicides

These chemicals were suspected of being a major cause of the decline of SAV in the Bay, but have been recently discounted by some as the direct cause of reduced plant populations. Although recent studies rarely isolated concentrations above 40 ppb, sampling was largely performed in main channels of tributaries and/or the Bay proper. When sampling was done at the heads of tributaries, isolated high concentrations of 100 ppb or more were recorded. Ephemeral pulses, single or multiple, could occur if rains followed herbicide application. Submersed macrophytes in tributaries could be exposed to high concentra-

tions of herbicides, yet these concentrations would escape detection due to dilution by water in the channels of the rivers.

It is probable that submersed plant beds are frequently exposed to at least low levels of herbicides, particularly in the upper portions of small streams and tributaries. No research has been done on sublethal stresses of herbicides on various phases of the life cycle of submersed vegetation. This research, therefore, concentrated on low-dose effects (those which would commonly be found in Chesapeake Bay tributaries) on the life cycle of an ecologically important submersed plant, Vallisneria americana.

CHAPTER II

THE EXPERIMENTAL ORGANISM

Vallisneria americana L. was chosen as the target organism for susceptibility to an herbicide, since it is a very desirable species of SAV. Ball (1965) reports use of it as waterfowl food in management practices in the southeast. As a fresh to low saline water macrophyte, it has significant importance to fish and benthic organisms in the upper reaches of the Chesapeake Bay, as Zostera marina does in the more saline portions of the Bay. Anderson (1980) has called Vallisneria a fresh water equivalent of Zostera. All parts of the plant structure are utilized for food by waterfowl and fish. In addition, its mere presence has distinct advantages, as previously outlined. Toxic effects and sublethal stresses from herbicides could cause more resistant and less desirable species, such as Myriophyllum spicatum, to competitively displace it.

Although Vallisneria is reported as a fresh water species, it does occur in low saline tidal rivers. Adult plants have been observed in as high as 10 ppt salinity (personal observation). The effect of this parameter on the total life history is not known.

Carbon (as $C^{14}O_2$) uptake rates have been shown to be reduced in V. americana with an increase in pH from 7 to 8 (Titus, 1980). This would seem to indicate that this species would not be found in highly alkaline bodies of water.

The bulk of research on environmental requirements and tolerances of Vallisneria has dealt with adult plants. Other "pheno-phases," particularly those dealing with reproductive events, have received little attention. Critical stages of development such as production of new growth from over-wintering tubers, leaf elongation, extension of lateral rhizomes or tuber production have not individually been tested for requirements or tolerances.

A brief review of the life history of Vallisneria growing in natural habitats of the Chesapeake Bay region is pertinent at this point. Vallisneria is a perennial species over-wintering in this geographic area as small tubers buried in the mud or sand substrate. By approximately mid-April when water temperatures reach about 15°C, germination of a single shoot from each tuber occurs. Growth continues until the shoot emerges from the substrate and begins photosynthesis. Root production also occurs during this time. Rapid production and extension of leaves continue until six to twelve leaves are produced per plant.

Vegetative reproduction via horizontal, below substrate rhizomes occurs during the following months, extending the plant bed. New shoots are produced at the nodes of the rhizomes, and the subsequent roots and leaves increase the biomass of each new plant.

In early August, the first evidence of sexual reproduction is observed, that being female flowers on extended peduncles. Submersed male flowers are produced on separate plants. Pollen is released to the surface and fertilization of the female flowers occurs. Fruit and seed production follows.

In the Chesapeake Bay area, August is the peak of biomass production for Vallisneria beds (Nichols & Anderson, 1978). From this

time growth slows, sloughing of old leaves occurs, and biomass declines. This is also the time of production of a unique rhizome which extends vertically into the substrate and produces a tuber for over-wintering. By the end of October most of the above ground vegetation has disappeared.

CHAPTER III

THE EXPERIMENTAL HERBICIDE

Since Atrazine is used extensively in the Chesapeake Bay area for control of weeds in corn fields, it was selected as the experimental herbicide. It is applied as many as three times throughout the growing season beginning in April. It may also be combined with other chemicals and used to control weeds in non-cropland situations.

Aside from the predominant use for weed control in cornfields, Atrazine is also used with other crops such as sugar cane, grass-seed crops, sorghum, pineapple and macadamia orchards, and conifer reforestation (Weed Handbook, 1979). In the past (early 1970s), Atrazine has been frequently used in aquatic weed control.

Chemistry of Atrazine

A brief summary of the chemistry of Atrazine is in order, if the potential potency to non-target plants is to be researched. Atrazine is a heterocyclic symmetrical nitrogen compound accounting for its designation as a s-triazine. Figure 1 shows the chemical formulation for this compound:

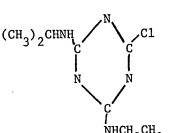


Fig. 1. Chemical structure of Atrazine, 2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine.

It possesses a molecular weight of 215.7, has a low vapor pressure (3.0 x 10^{-7} mm Hg at 20 C), and has solubility in water at 27 C that is pH dependent (31 ppm at pH 3, and 35 ppm at pH 7).

Field Application

Atrazine can be applied at three different times during the growing season. It can be used (1) prior to planting for control of weeds such as quackgrass, (2) after planting of the crop seeds, as a pre-emergent (in this capacity it prohibits germination of most broad leaf weeds), or (3) as a post-emergent combined with oils or surfactants as adjuvants for improved control of annual grasses. Most commonly, this herbicide is applied as a pre-emergent and application is followed by one cultivation (McEwen & Stephenson, 1979).

Methods other than direct application of the herbicide to the soil are employed. Atrazine formulated on inert clay particles, can be made into a suspension and applied as a spray. With this method the potential for large amounts of Atrazine to reach non-target areas through drift and atmospheric fallout is considerable (Glotfelty, 1983).

Wind can carry the herbicide to adjacent areas. When the pesticide is applied in granular form, the drift is reduced to almost zero due to the relative heavy weight of the granules. Spraying, however, can result in as much as 50% being lost. The amount lost can become quite high in those situations where it is applied to the canopy of an orchard, since the spray is directed upward. Large amounts can also be subject to draft when aerial spraying is done. The distance the droplets travel is inversely related to their size. McEwen and Stephenson (1979) report most applications of pesticides are made with

droplet size in the 30 to 300 μ range, but all applications have some droplets as small as 5 μ. Droplets as large as 20 μ can be carried over two miles in a small air current of 3 mph and this distance is increased to 21 miles if the size is reduced to 2 μ (Akesson & Yates, 1964).

Relative humidity also affects droplet size. Brann (1965) did a study relating decreased relative humidity to high water evaporation from the droplet released from the spray orifice. This evaporation decreased the droplet size and increased drift. The amount of pesticide present in this drift carried by the droplet varied inversely with the droplet size. He found that the soil having the most contamination is usually a narrow area adjacent to the acreage being sprayed. Another study (Laubscher et al., 1971) demonstrated that a concentration as high as 10 ppb of a pesticide could be isolated 10,000 meters from a treatment area. Drift of aerial application and the potential fallout into the tributaries of a watershed is quite large whenever agricultural planting is done either adjacent to, or within the drainage area of creeks and streams, which join tributaries of a large water body.

Persistence Characteristics

Once the herbicide is applied to the soil, its persistence is dependent upon a number of environmental factors such as soil moisture, soil temperature, soil pH, and degradation.

Soil Moisture

Hall et al. (1972) reports a study performed in the spring where Atrazine was applied at a normal rate of 2 lb/acre to a Pennsylvanian soil consisting of silty clay loam. As much as 15% remained after one year.

McCormick and Hiltbold (1966) demonstrated that persistence of Atrazine is decreased in warm, moist, low adsorptive soils. The fluctuation of Atrazine adsorption in relation to soil moisture was also studied by Green and Obien (1969). They found that more herbicide is usually adsorbed when soils are devoid of moisture. This decrease in soil moisture favors adsorption, since H_2O molecules are polar, and they compete with the herbicide for adsorption sites, which in turn would cause more herbicide to go into soil solution.

When rainfall is abundant, residual Atrazine is decreased in the soil (Burnside et al., 1971), but potentially can be included in runoff into a tributary.

Temperature

In addition to lack of rainfall, low temperatures may also increase persistence. A decrease in temperature from 30 ° to 15 ° C increases the halflife of Atrazine from two months to six months (as demonstrated in a study reported by Brown (1978). This inverse relationship to temperature can allow Atrazine to remain active in the soil for longer periods of time, if applied in early spring when cold temperatures are still prevalent. This herbicide would then be subject to runoff, posing potential adverse environmental consequences.

Under normal conditions when Atrazine is applied as a preemergent at standard application rates, its usual persistence is three to twelve months in temperature zone soils (Klingman & Ashton, 1975). Broan (1978) reports an average of eight months to decrease it to 75% of its original amount, which is a slightly longer time than reported by Hall as cited previously. McEwen and Stephenson (1979) reported that an

application rate of Atrazine of less than 2 lb/acre, it was usually degraded to nonphytotoxic levels at the culmination of the growing season, but at rates exceeding 2 lb/acre, residues were carried over into the following growing season.

Soil temperature can also affect the adsorption of Atrazine onto soil particles. Adsorption is an exothermic reaction in which H-bonds and ionic bond formation release heat (Bailey & White, 1964). With increased soil temperatures, bonds can be broken and desorption of herbicide molecules results. Greater soil temperature results in greater soil solubility which further shifts the sorption equilibrium. Harris and Warren (1964) demonstrated that adsorption of Atrazine onto organic matter is not temperature dependent, but adsorption onto soil minerals is; being less by a factor of 3 at higher temperatures. Table 1 summarizes their results.

TABLE 1

ADSORPTION OF ATRAZINE ONTO ORGANIC (MUCK) SOILS AND SOIL MINERALS

Muck Soil (pH 5.6)			Bentonite pH (8.5)	
Temperature	0	50	0	50
Adsorbed (%)	42	40	41	12

Roeth et al. (1969) concluded that degradation occurred two to three times more quickly in top soils, when compared to subsoils. He also noted an increase in degradation of two to three times with every 10°C increment in temperature from 15°C to 35°C.

Soil pH

Another physical parameter that affects adsorption of Atrazine onto soil particles is pH. Adsorption is usually higher in more acidic soils. A very slight increase in soil acidity can convert negatively charged anions to uncharged or even positively charged cations, which would consequently increase adsorption since the adsorption site on the clay particles is negatively charged. If soil conditions become extremely acidic, hydrogen ions successfully compete for negative adsorption sites, and herbicide adsorption is decreased. If, on the other hand, soil conditions become strongly alkaline, adsorption is low (especially for herbicides that are anionic) since they are repelled by the negative bonding site.

The overall relationship of adsorption of Atrazine to pH of the soil moisture and temperature is summarized in table 2. This study, conducted by McGlamery and Slife (1966), illustrated the lack of relationship between water solubility of this herbicide and adsorption (increased water solubility does not necessarily increase adsorption), since the Kd values change only slightly (actually decrease) as the temperature is increased from 10 °C to 30 °C. However, the percentage adsorbed rises dramatically as the soil becomes more acidic.

This physical characteristic of Atrazine had been previously demonstrated by Talbert and Fletchall (1965) when soils were ranked from high to low pH values, Atrazine adsorption followed this ranking with the least adsorption occurring at high pH values, and the greatest adsorption occurring at low pH values.

TABLE 2

PERCENT ADSORPTION OF ATRAZINE ONTO SOIL AT DIFFERENT TEMPERATURES AND VARYING pH

	Temperature		
Soil pH	10 C	20 C	30 C
3.9	12.9	12.6	10.4
5.3	8.0	8.2	6.8
8.0	66.2	6.3	4.4

SOURCE: Brown, 1978.

Degradation

Biological. Biological degradation is inversely related to the amount of adsorption onto soil particles, as well as directly related to the type of organismal activity in the soil; the major groups being bacteria, fungi, and algae. Microorganisms are unable to degrade Atrazine if it is adsorbed onto soil particles (McEwen & Stephenson, 1979).

Whatever fraction of the herbicide that remains in soil solution after the adsorption-desorption equilibrium is reached with soil colloids, is immediately available for use by those microorganisms which can easily adapt to it as an energy source (McEwen & Stephenson, 1979). These organisms would then rapidly increase in number, with the limiting factor being the total degradation of the herbicide. When this energy source had been depleted, their numbers would decrease. If, however, the herbicide is only slightly soluble, or strongly adsorbed onto soil particles, or the microorganisms are unable to adapt to it as an energy source, the degradation does not proceed by a logarithmic or sigmoid curve, characteristic of degradation by soil organisms, but rather follows first order kinetics of a linear curve, indicative of degradation by physical or chemical means. Atrazine is a moderately persistent

herbicide and follows, as Harris (1967) discusses, first order kinetics. This lack of biodegradability of Atrazine is partially due to its chlorination, since degree of chlorination is directly related to degradation (Harris & Waren, 1964; Harris, 1967; Kaufman & Kearney, 1970).

When degradation does take place, it is a slow process. Harris (1967) comments that adsorption, desorption, and solubility factors combine to prevent all but a small percentage of Atrazine to be available for degradation at any given time. The microbes that attack Atrazine are very specific for that herbicide. This phenomenon was illustrated by Butler et al. (1975) in a study in which twenty-one species of planktonic algae were incubated with 1 ppm of Atrazine for two weeks. At the end of this time, the degree of degradation was measured. Only 12 to 21 species of algae grew in the presence of Atrazine, and none were able to degrade it, in comparison to 2,4-D, which was degraded by all cultures of algae in a similar experiment.

When degradation by soil bacteria and fungi does occur, they are able to dealkylate Atrazine, but are unable to cleave its triazine ring, unless it is first chemically converted to the hydroxy analogue. Wolf and Martin (1975) proposed cyanuric acid as an intermediate in this slow process of ring cleavage yielding a final product of CO_2 and NH_3.

Chemical. Interwoven with microbial degradation is chemical degradation, which plays a critical role in the complete breakdown of Atrazine. Microorganisms are unable to proceed in some stages of the breakdown process unless the intermediate products are first chemically converted. Unlike biological degradation, adsorption onto soil

particles increases chemical degradation, as do low pH levels, and increases in soil temperature.

Armstrong and Chesters (1968) point out that the chemical hydrolysis of Atrazine to its 2-hydroxy analogue is slowly catalyzed by adsorption onto soil particles and that chemical degradation increases at low pH levels. This finding would account for the principal metabolite of Atrazine degradation found in the soil being the hydroxy analogue, which is fairly stable against breakdown by microorganisms and is a nonphytotoxic product of chemical degradation. This conversion of Atrazine to the hydroxy analogue increases with the soil temperature. The further breakdown of hydroxy-Atrazine proceeds faster under anaerobic conditions, as opposed to aerobic conditions.

Photodegradation. Ultraviolet radiation causes chemical degradation of Atrazine on soil surfaces. This process, referred to as photodegradation, occurs rapidly in full sunlight and at summer temperatures. Jordon et al. (1964, 1965) found that the loss of Atrazine after UV light exposure decreases with time. The degradation that occurred to the surface layer of the herbicide acted to protect the underlying herbicide from further photodegradation. It was further discovered that Atrazine was photo decomposed (being hydroxylated to 2-hydroxyatrazine), when irradiated in water, at wave lengths less than 300 nm. However, when the irradiation was performed in aluminum dishes, considerable photodecomposition occurred at 311 nm (Jordon et al., 1970).

When Atrazine was exposed to full sunlight in spring, it was found to be photodecomposed by 45% as compared to 16% in shade. Summer temperatures increased the loss from 65% to 80% (Comes & Timmons, 1965).

Therefore, the lack of direct sunlight and cool temperatures favor the persistence of Atrazine.

Although photodegradation is not a major pathway of disappearance of Atrazine, overall degradation whether it is biological or chemical, along with adsorption, influence the availability for transport to non-target areas.

Transport from Sites of Application

Volatilization

Although Atrazine possesses a low vapor pressure, transport can occur by volatilization at times of application when intense sunlight heats the soil surface. Kearney et al. (1964) observed an increase in volatility of Atrazine of 50% for the 10 degree rise in temperature from 35° to 45°C. The moisture content of the soil also increased the volatilization of Atrazine both in amount and rate, with the light textured soils having the most volatility.

Residues of Atrazine were found in rain a mile away when fields were treated with Atrazine three weeks earlier (Cohen & Pinkerton, 1966). Particulate matter with adsorbed Atrazine contained in precipitation or as direct fallout presents a potential environmental hazard to non-target areas (Glotfelty, 1983). If climate conditions are not such that the Atrazine is volatilized, then it is available for translocation by leaching or runoff.

Leaching and Runoff

The amount of leaching of Atrazine is dependent upon the amount of rainfall. The more soluble or poorly adsorbed an herbicide is, the more easily it is leached during times of high rainfall, thus necessita-

ting repeated applications. The net result is contamination of water tables. Although traces of Atrazine have been isolated in drinking water, studies have shown that little is lost to leaching.

In addition to leaching, pesticides which are least adsorbed are subjected to movements laterally with water that is running off of agricultural fields. Soil particles are also transported as a result of erosion. If an herbicide, such as Atrazine, is not biologically or chemically degarded, it may be adsorbed onto soil particles and is subject to erosion. In general, the total loss of Atrazine is directly related to rainfall during a growing season, which is the critical factor in runoff losses. Axe et al. (1969), in an experiment performed in Texas, found 40 ppb of Atrazine in runoff water. A study conducted by EPA (1971) demonstrated that the transport of a majority of the herbicide was the result of attachment to soil particles. Keeping in mind that 4 billion tons of sediment are produced yearly by erosion (McEwen & Stephenson, 1979), this potentially carries a significant amount of Atrazine.

Whether Atrazine is transported via adsorpted particles in runoff water laden with sediment, or volatilized and transported long distances with winds, environmental concerns are raised due to its potential threat to nontarget areas such as near shore shoal containing submersed vegetation.

CHAPTER IV

ABSORPTION AND TRANSLOCATION OF ATRAZINE BY PLANTS

Once an herbicide, such as Atrazine, has been transported into a water column, it is now available to be first absorbed and then translocated to its site of action in the plant where it is potentially phytotoxic. This process cannot only be affected by both chemical and physical properties of an herbicide, which will influence the rate and pathway, but also by various environmental factors, such as temperature and light. In addition, plant characteristics and site of action can influence the quantity of the chemical absorbed into the plant and its eventual pathway.

In order to better understand absorption and translocation it is critical that the anatomy and physiology of a SAV be considered. The cuticle, if existent, is very thin. Presence of a cuticle would be detrimental to an aquatic plant in that it limits gas exchange. Stomates, if they occur, are functionless. Most aquatic plants are monocots, and they have poor cellular differentiation. No definite pallisade tissue exists in the leaf compared to terrestrial plants, in which the pallisade and mesophyll are well developed. Emergent or floating leaves differ from submersed leaves and are more morphologically and physiologically related to terrestrial plants. Elodea, for example, has little cellular structure, but the lacunae are well developed.

The vasculature of the stem of SAV differs from terrestrial plants. Xylem transport in vascular land plants is accelerated via the transpiration stream. In aquatic plants the plasmodesmatal connections contribute the major route for internal transport. The xylem tracheal elements are usually poorly lignified since the water lends support to the plant. The vascular tissue is much reduced, and condensation into a central vascular cylinder usually cocurs (Sculthorpe, 1967). Elodea as well as Potamogeton spp. transport through the symplast (living cells). Within leaves, transport can occur in any direction depending upon the concentration gradient. Because of the aquatic environment, material can also be lost by diffusion from intercellular spaces, since it is in continuous aqueous contact with its surroundings.

Roots of SAV also have the vasculature reduced. In some plants the roots are so poorly developed that they do not have a physiological function other than some ion uptake (Schulthorpe, 1967). The xylem elements of Elodea, Najas, Ruppia, Vallisneria, Zannichellia, and Zostera are very reduced. There is no thickening in the few tracheids that they possess though the phloem is well developed.

Aldrich and Otto (1959), in one of the earliest studies with herbicides, recognized that conclusions drawn from investigations of herbicide movement in terrestrial plants was not necessarily applicable to SAV because (1) water surrounds the entire shoot and leaf portion of the plant, usually allowing uniform contact with the dissolved chemical, and (2) vascularization is minimal in aquatic plants. Sculthorpe's classic book (1967) was not yet published at this time to aid them with a better understanding of the vascular nature of SAV, which led them to

assume that the anatomy of all SAV is similar. It has now been shown to vary considerably.

In general, translocation is through apoplastic, symplastic or a combination of both systems. Although translocation of Atrazine occurs predominantly via the apoplastic system, all herbicides are translocated to some degree in both systems (Ashton & Crafts, 1981).

The apoplastic system consists of intercellular spaces, cell wall, and mature xylem. This is considered to be non-living, compared to the symplasm. If Atrazine is absorbed by the roots, apoplastic movement would result in transport via the transpiration stream. If absorption occurs through the leaves, the Atrazine will usually remain in the leaf.

Since the vascular tissue is greatly reduced in most SAV, an herbicide such as Atrazine, which displays predominant apoplastic movement, would not be expected to be transported upward if it is present in the sediment. However, due to a lack of reduced cuticle in a submersed plant, if Atrazine is present in the water column, the leaves should readily absorb this herbicide, but exhibit little translocation.

This phenomenon was observed by Forney and Davis (1981), who looked at differences in effect of Atrazine applied to roots and/or shoots of P. perfoliatus and Elodea. When applied to the soil and not to water, concentrations as high as 1,000 ppb did not inhibit growth, compared with concentrations of 100 to 1,000 ppb in water which inhibited growth 18% and 49%, respectively, for Potamogeton. For Elodea, concentrations of 32 and 320 ppb in water inhibited growth 21% and 65%, respectively, but in soil, growth was not inhibited at 1,000

ppb. If Atrazine ws present in both soil and water, no increase in inhibition was observed.

Since Elodea and Potamogeton, like Vallisneria, have much reduced transpiration streams, little apoplastic translocation should occur. The results of the study by Forney and Davis; namely, that no inhibition occurred when Atrazine was present only in the soil, appears to reflect the lack of translocation due to poorly developed xylem tissues.

The above studies dealing with SAV demonstrate that for Atrazine (1) absorption occurs readily through the foliage, (2) little downward or basipetal movement occurs, (3) root absorption plays a minor role in translocation because of a reduced transpiration stream in most SAV, and (4) when apoplastic movement does occur in aquatic plants, they are either emergents or possess floating leaves and/or a well developed transpiration stream.

CHAPTER V

MODE OF ACTION OF ATRAZINE

Susceptible Species

The site of action of Atrazine in the cell is the chloroplast. It acts as a potent inhibitor of the light phase of photosynthesis (specifically, the Hill reaction) in susceptible plants. It appears to interfere at or close to Photosystem II in the light reaction. This would not only suppress oxygen evolution by the plant, but would further alter its physiology by preventing the formation of $NADPH_2$, and the transport of electrons that eventually generate ATP (Bidwell, 1979). CO_2 would thus be prevented from being fixed and reduced to carbohydrates, which in turn should be reflected in the cessation or slowing of growth of the plant.

It was assumed (Bishop, 1961; Van Overbeek, 1962; Zweig, 1969) that the triazines were inhibiting PSII somewhere between the primary electron acceptor Q (for quenching of fluorescence) and PQ, plastoquinone. Q was thought to be a tightly bound molecule of plastoquinone.

Van Overbeek (1962) used fluorescence as an indicator of herbicidal inhibition of the Hill reaction. He stated that the re-emission of light is inhibited by the triazines. The electrons that are captured by the electron acceptor (Q) are prevented from being shuttled down the electron transport chain and are just held. This causes the chlorophyll to eventually become oxidized, since the electrons are neither returned

nor replaced by the OH^- ions. Chlorosis, a bleaching from oxidation, follows.

A study cited in Moreland (1980) shed some light on the location of the binding site. In this investigation, the proteinaceous component located over Q was removed by a mild trypsin digestion. Sensitivity to electron transport inhibitors such as Atrazine was decreased and there was a loss of two protein bonds of M.W. 32,000 and 37,000. It was felt that the herbicide was interacting with the allosteric shielding protein (a macromolecule whose reactvity with another molecule is altered by combination with the third molecule) and not directly binding with the electron carrier. This binding would disrupt the reaction kinetics or cause conformational changes that might interrupt electron flow. Recent studies (Coydell, 1983; Jensen, 1980) indicate that there are two quenchers, Q_L and Q_H.Q_L in current literature is sometimes referred to as Q or PQ1. The PQ1 or Q_L is the first stable electron acceptor. This $PQ1^-$ now reduces the PQ2 which in turn shuttles the electron to the pool of plastoquinone. PQ1 and PQ2 have identical spectroscopic properties (Mathis & Paillotin, 1981).

Gardner (1981) investigated the disruption of the above electron transfer by Atrazine, using a radiolabeled analog of Atrazine. His theory is that a carrier, B (this B would be equal to the PQ2), is normally reduced by an electron from the primary electron acceptor PQ1 and in turn donates an electron to the plastoquinone pool (PQ) that connects PSII and PSI. When the triazine binds, it acts at the second electron carrier (this "B") on the reducing side of PSII. Gardner believed that this carrier was a type of quinone molecule bound to a specific polypeptide in the reaction center. He further found that the

radioactivity was always associated with a polypeptide of M.W. 32,000. His conclusion was that this was the triazine receptor. Therefore, when the triazine binds, PQ still accepts the electron, but B (PQ2) is no longer capable of accepting the electron to shuttle it on.

Tolerant Species

At moderate levels, Atrazine is a very potent broad-leaf weed killer, due to the plant's inability to metabolically detoxify this chemical. Terrestrial monocots, such as corn and many annual grasses, have a high to moderate tolerance, respectively, for Atrazine. They degrade the triazines by one of three methods: (1) hydroxylation at the 2-carbon atom of the ring with displacement of either chlorine, methoxy, or methylthio group (in other triazines); (2) dealkylation of the side chains; and (3) conjugation, usually with glutathione. Ring cleavage sometimes occurs, but if it does it is a very slow process (Montgomery & Freed, 1961). Davis et al. (1965) used ring labelled C^{14} and found less than 2.5% $C^{14}O_2$ after one week.

Hydroxylation

Initially, the ability to convert the triazines to the hydroxy derivative was thought to be correlated to the amount of benzoxazinone, BOA, that was present in a plant. However, some plants such as grain sorghum contain no BOA, but degrade triazines to hydroxy derivatives, all of which are water soluble metabolites. Subsequent research has shown that resistance in corn involves conjugation as the predominant pathway.

Dealkylation

If either or both of the side chains of the triazines are dealkylated an amino group remains. These metabolites are found in the chloroform fraction and are still slightly phytotoxic, although the potency of the parent triazine has been substantially reduced (Ashton & Crafts, 1981).

Conjugation

Most resistant plants have conjugation as a predominant pathway (Shimabukuro et al., 1970). The triazines are predominantly conjugated with glutathione or glutemylcysteine (referred to as GS-Atrazine by Lamouteaux et al., 1972). The degree of resistance is correlated with activity of the enzyme glutathione - s - transferase. Furthermore, if any unchanged Atrazine is translocated from root to shoot in the xylem, it is rapidly converted by the enzymes to GS-Atrazine. In the root, the enzymatic reaction does not seem to occur. Where it is present, it appears to out-complete the BOA for the substrate molecule of Atrazine (Shimabukuro et al., 1971).

In general, plant resistance is closely correlated with its ability to degrade triazines (Shimabukuro, 1967). The rate has to be such that the herbicide is degraded before any toxic accumulation can occur and degradation must produce a non-phytotoxic chemical. Dealkylation can be coupled with ring hydroxylation if BOA is present in the plant. If both pathways operate, then the rate is critical in determining resistance, e.g., corn vs. wheat. Shimabukuro felt that the dealkylation was operable in most higher vascular plants and the rate and accumulation of partially dealkylated Atrazine, which is somewhat

phytotoxic would account for the gradation of resistance seen in plants with intermediate susceptibility. Since conversion of Atrazine to hydroxy derivatives leads directly to a non-phytotoxic metabolite, this pathway is more efficient.

V. americana, although a monocot, does not possess this tolerance displayed by its terrestrial and estuarine counterparts such as corn and Spartina spp., respectively. In addition, unlike terrestrial plants where the roots are the primary uptake site, absorption in this aquatic plant, which lacks a cuticle, occurs mainly through the leaves. Therefore, even small ambient concentrations in the water could be stressful.

CHAPTER VI

MATERIALS AND METHODS

There were three distinct, but interrelated, aspects of this research: (1) field studies associated with the various life-history events in Vallisneria americana, and collection of experimental plant material; (2) design and construction of laboratory microcosms by which the long-term studies of Vallisneria could be made under controlled conditions; and (3) short-term experiments for evaluation of photosynthetic and respiratory rates.

Field Studies

It was important to establish the timing of various aspects of the life-cycle of Vallisneria under field conditions prior to and during microcosm studies, since one of the primary goals of this research was to determine sublethal effects of Atrazine on each of the phases of the life cycle. Timing of events in microcosms to simulate those under field conditions was also important for determining dose-response relationships.

The following sites were utilized for monitoring growth phases of Vallisneria and water quality parameters associated with healthy beds of plants.

1. Susquehanna River at Havre De Grace, Maryland.
2. Magothy River, northside of Dubbins Island, Maryland.
3. Saltpeter Creek at Crane generating station, Maryland.

4. Potomac River at Route 301 Bridge, Virginia.

5. Goose Creek and Port Tobacco River, Maryland. This was the primary collection site for plant material, substrate and water for microcosm studies.

At all of these sites the following parameters were monitored:

1. Salinity; water temperature; conductivity: Yellow Springs Instrument Company (YSI) salinity, conductivity and temperature meter, Model 33.

2. Substrate temperature: YSI Tele-Thermometer, Model 46 TUC.

3. pH: Markson, Model 92 meter.

4. Dissolved oxygen: YSI Model 57 oxygen meter.

Continuous Flow Culture Chamber (Microcosm)

A unique system had to be designed to study the different phenophases of V. americana over an extended period of time. Other systems (Correll & Wu, 1982; Forney & Davis, 1981) had limitations that would not allow the design to be adaptable to a study which encompassed all phases of the life cycle of an SAV, notably: (1) a system which would ensure dilution of the chemical prior to circulation in the immediate vicinity of the plant, (2) continuous circulation of the water around the plants, (3) sufficient depth of the water column to allow for leaf length growth approximating that of field conditions, (4) sufficient depth of the substrate to allow for tuber formation, (5) ample surface area of the substrate to achieve a large population sample and allow rhizome formation, and (6) an ability to control and monitor physical parameters such as temperature, pH and nutrients without any disruption to the plants.

The design developed was as follows: two 18 gallon aquaria (Odell), measuring 51 x 27 x 52 cm were connected in such a way as to maintain a continuous circulating water supply between them. This arrangement constitutes one microcosm. Six microcosms (12 aquaria) were utilized. Each microcosm had water circulation maintained by a Cole-Palmer polyethylene centrifugal pump, Model 7000 (Betts et al., 1967), regulated by a variable transformer in such a way as to ensure adequate circulation, but to prevent resuspension of the sediment into the water column.

Each microcosm consisted of one experimental aquarium and one dosing aquarium. Each experimental aquarium contained 12 cm of sediment, 18 Vallisneria plants, and filtered (Whatman No. 1 filter paper) river water of 1-2 ppt salinity, all obtained from the Goose Creek collecting site. The aquaria were fitted with a plexiglass top to reduce evaporation and limit contact with the atmosphere. Holes were drilled in the plexiglass top to accommodate an inlet tube, an outlet tube, 2 water level equilibration tubes, and a multipurpose opening for removal of sloughed material and oxygen monitoring.

Each dosing aquarium contained filtered river water (salinity of 1-2 ppt), a clear plexiglass top that had holes drilled for a heater-thermostate, inlet tube, outlet tube, 2 water level equilibration tubes connected to the experimental aquarium, and a small opening for a hypodermic syringe for dosing. The total volume of the system was 117 liters.

Lighting for plant growth was supplied by fluorescent Vita-Lites^{R}, which most nearly simulated natural sunlight (data from Duralight Corporation and Smithsonian Radiation Lab, personal communica-

tion). A photometer (Model LI-185, Lambda Instruments Coo), was used to measure light intensities at the surface of the water, and at the top of plant leaves. Light levels were maintained at 64 μ E m^{-2} sec^{-1} previously determined as an optimum level by preliminary experiments in the microcosms. A light regime of 13 hours, similar to that of field conditions, was maintained. A circulation flow of approximately 4 liters per minute was maintained to ensure adequate dilution of the herbicide prior to contact with the plant and uniformity of physical parameters such as water temperature and pH. Use of a Beckman model 0260 oxygen meter and probe attached to a recorder provided oxygen concentration measurements over 24 hour periods to verify that a diurnal curve had been maintained in the microcosms. Additionally, measurements were made periodically to determine if ample dissolved oxygen was being maintained and did not become a limiting factor with time. Oxygen probes connected to the digital oxygen meter were used, and readings were verified by Winkler titration (Standard Methods, 1976).

Trials of 4 to 6 weeks duration were made for each of three stages in the life cycle of V. americana. Plants were randomly collected from the field site at the beginning of each trial and transported to the laboratory and immediately placed in the experimental aquaria. The timing of field collections combined with the laboratory environment provided necessary ques for completion of that stage of the life cycle of the plant as it occurred in the field.

Prior to the trials with Atrazine, baseline data of the different phenophases of V. americana were collected. This data served as a reference point for subsequent herbicide exposure.

For the toxicity studies, a series of six microcosms were simultaneously monitored. One served as a control, the other five had various concentrations of herbicide in the water column. Technical grade Atrazine (96.5% purity) supplied by the manufacturer, Ciba-Geigy, was used for all dosing experiments. The appropriate amount of Atrazine to achieve the desired concentration in the microcosms was first dissolved in 3 ml of methanol, which served as the carrier (after Bowman, 1981). After the plants had a 14-day acclimation period within the microcosm, all microcosms were dosed. A hypodermic syringe was used to introduce Atrazine just below the surface of the water column in the dosing aquarium, to ensure dilution to the desired concentration, before contact with the plants was made. Only the carrier was added to the control microcosm. Sloughed material was periodically removed from all experimental microcosms so that it could be weighed before deterioration. Water samples were taken at days 0, 1, 3, 7, 15, and 30, after dosing and submitted to EN-CAS Analytical Laboratories, Winston-Salem, North Carolina, an independent laboratory for verification of dose levels.

At the conclusion of each microcosm experiment, the total wet-weight biomass of all plants, as well as individual leaf lengths were recorded. Vegetative growth and sexual reproduction were monitored throughout the duration of each experiment, and quantified at the end.

A separate set of holding tanks was used to acclimate Vallisneria brought in from the field. Plants were collected from an area encompassing the northern shoreline at Goose Creek and extending northward into the western shoreline of the Port Tobacco River. These tanks were maintained at conditions identical to that of experimental

microcosms. Vita-Lites^{R} provided a light-dark regime similar to that of field conditions.

The Gilson Differential Respirometer, Model IGRP-20

This instrument was utilized to establish photosynthetic and respiration rates of Vallisneria as a consequence of short term (4 hours) exposure to Atrazine. For each "run" 20 volumometers were filled with 7.5 ml of filtered Chesapeake Bay water, gathered from the plant collection sites. Salinity of the water was the same as the microcosms and holding aquaria; namely, 1 to 2 ppt. This reflected field conditions where V. americana was gathered. Temperature of the water was maintained at 25°C, the same as the microcosms and holding aquaria. The center well of each volumometer contained a 20% solution of NaOH to absorb CO_2 produced during respiration. Two of the flasks were left empty of plant material to serve as controls, and two containing plant material were covered with foil to measure only respiration. The remaining 16 flasks contained an apical section of leaf blade, and net photosynthesis and respiration were measured. This totally closed system was constantly agitated and subjected to an acclimation period of 10 minutes prior to closing, as well as to a constant temperature and light regime (25°C and 275 microeinsteins m^{-2} sec^{-1}). Changes in gas volume resulting from metabolic activity were recorded every half hour for 4 hours to ensure measurements within the photosynthetic and respiratory plateaus.

At the conclusion of each experiment, plant material was removed, washed, and dried at a constant temperature at 104°C for 24 hours to a constant weight. Rates of oxygen evolution are expressed as

μl O_2/mg dry weight. Two trials at each of the selected doses were performed. Representative samples of all doses were submitted to the same independent laboratory for verification of concentrations used. These doses were found to be accurate within 1 to 2 ppb.

The guidelines set forth in the EPA, Chesapeake Bay Program, Quality Assurance Manual are followed for equipment, glassware, analysis, etc. These include:

1. Interlaboratory evaluation of data. Samples are submitted to an independent laboratory for analysis.

2. Calibrated water quality equipment dedicated to the project.

3. Routine water analyses are made using only standard methods.

4. Demineralized water is used for all dilutions, made in a glass distiller.

5. All data analyzed through use of university computer facilities. Programs exist for statistical analysis. A program has been written to analyze the Gilson respirometer data.

CHAPTER VII

RESULTS

Prior to dosing with herbicides the behavior of Vallisneria was monitored through a growing season in microcosms and in the field. All aspects of the life cycle discussed previously were observed in laboratory microcosms as well as in the field. Plants collected early in the growing season grew vigorously and attained leaf lengths up to one meter in a six week period. Extensive rhizoming occurred, in some cases more than doubling the number of plants in a microcosm (18 to 39).

Table 3 illustrates the increase in number of plants for the control tanks during a six week period early in the growing season. The percent change in each microcosm was always greater than 100. The plants had ample surface area to allow for horizontal rhizoming of 10 to 15 cm to produce each additional new plant.

TABLE 3

THE INCREASE IN NUMBERS OF V. AMERICANA PLANTS IN CONTROL MICROCOSMS BETWEEN MAY 21 AND JULY 2, 1982

Control	No. Plants Initial	Final	Percent Change
1	18	38	111.1
2	18	37	105.5
3	18	38	111.1
4	18	39	116.7

The increase in total biomass for these same control microcosms is depicted in Table 4. Growth, expressed as a percentage of initial biomass, increased from 78% to 139%. Both male and female flowers were produced at about the time this phenomenon occurred under natural conditions. The peduncles of the female flowers grew extensively to over one meter in length and floated on the water surface.

TABLE 4

THE INCREASE IN BIOMASS OF V. AMERICANA PLANTS IN CONTROL MICROCOSMS BETWEEN MAY 21 AND JULY 2, 1982

Control	Biomass (Gms.) Initial	Final	Percent Change
1	20.1	35.7	77.6
2	18.1	36.5	101.7
3	19.9	46.2	132.2
4	19.5	45.6	138.8

A critical stage in the life cycle is the production of vertical rhizomes from August through September from which over-wintering tubers are formed. This growth phenomenon was also observed in the laboratory microcosms during the same period as in the field. Over seventy tubers were produced in the control microcosm.

Oxygen concentrations monitored over a 24 hour period showed diurnal fluctuations. Maximum concentrations of near saturation were always reached between 2:00-4:00 P.M. showing a few-hour lag from the period of maximum photosynthesis that occurs around 11:00-12:00 A.M. Minimum values of 6.7-7.0 ppm were reached at 5:00-7:00 A.M. Although the lights were activated at 6:30 A.M., oxygen concentrations did not begin to rise until 7:00-7:30 A.M.

The positive results of the microcosm design, as indicated by successful duplication of life history events of Vallisneria in the laboratory, allowed for the beginning of dose-response experiments.

Effect of Various Concentrations of Atrazine on Metabolic Rates, Growth, and Reproduction

Research on the effect of various concentrations of Atrazine on selected parameters of production, growth, and reproduction was conducted during the 1982 growing season and replicated during the 1983 growing season. The growing season was divided into three "segments" based on the life history of V. americana observed in the field under natural conditions.

Segment A. - Early growing period (mid-April to early July). Characterized by rapid leaf growth, extensive rhizome growth, and addition of new plants by vegetative reproduction. All plants are in the "juvenile" growth phase.

Segment B - Mid-season growing period (early July to mid-August). Characterized by maturation of leaves, continued production of new leaves and new plants from rhizomes, and beginning of sexual reproduction. This would be a transition period from "juvenile" to the "mature phase" of growth.

Segment C - Late season growing period (mid-August through early October). Characterized by deterioration and sloughing of older leaves. Much reduced new leaf and new plant production. Maturation of male and female flowers and pollination. Fruit matures near the end of this period. Production of vertical rhizomes and tubers. This would be a transition period from the "mature" to the "senescent" phase of growth.

Effect of Various Concentrations of Atrazine on Photosynthetic and Respiratory Rates

Photosynthesis

Short-term relationships between photosynthesis and Atrazine dose were examined to try to determine the time interval between dose and first measurable effect of various concentrations of Atrazine. Experiments were conducted for a four hour period with readings each one-half hour. For the control and each dose of Atrazine tested, a total of 180 data points, representing 16 replicates, were averaged and presented as gross photosynthesis: micro-liters of oxygen produced per milligram of dry weight. Figure 2 illustrates a bar plot of Atrazine dose related to oxygen evolution for the June and August 1983 experimental periods. Each bar represents the average of 180 data points. There appears to be a bell-shaped curve, which may indicate some stimulation at low concentrations.

Figure 3 illustrates time interval plots of oxygen evolution for the June and August experimental periods. Data for the control, 16, 32, and 64 ppb doses of Atrazine are shown. All curves show a sharp initial increase in oxygen concentration after flasks are closed to the atmosphere. Moreover, there is a general trend downward for all doses approximately one hour after dosing. The curves of the dosed flasks generally show a lag in the reduction of oxygen evolution compared to the control flasks.

Respiration

Data on plant tissue respiration and Atrazine dose were recorded in order to correct for this factor in flasks measuring oxygen evoluton

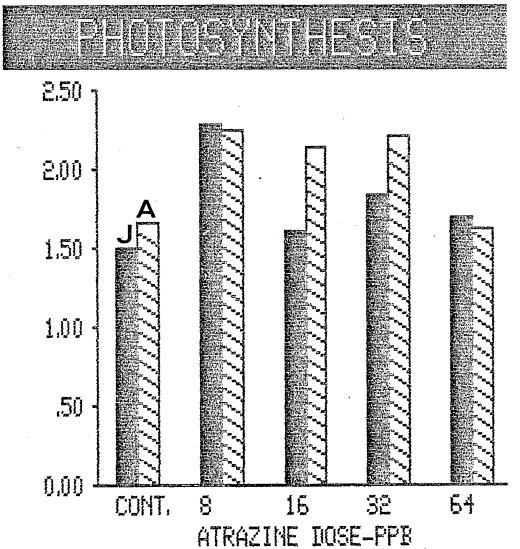


Fig. 2. Plots results of short-term experiments relating average oxygen evolution (Microliters/½ hr) to Atrazine dose for the June (J) and August (A) 1983 experimental periods.

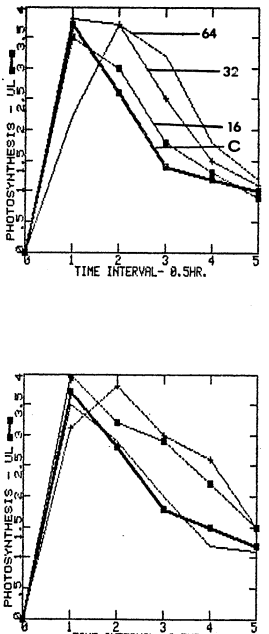


Fig. 3. Plots results of short-term experiments relating oxygen evolution to time during each of the experimental runs on the Gilson respirometer. The top plot is for June and the bottom for August 1983.

rates. In this way a gross photosynthetic statistic was obtained. Figure 4 shows average oxygen consumption for control and dosed flasks. The depression in June at a concentration of 8 ppb should be noted, as well as the apparent stimulation in August at the same dose. Data for June, after the depression seen at 8 ppb, approached that of the control. In August, the stimulation seen at 8 ppb tapered off and at 32 ppb fell below that of the control, before approaching a level equal to that of the control.

Effect of Various Concentrations of Atrazine on Leaf Growth and Biomass Production

Leaf growth

Virtually all of the above substrate growth in V. americana is reflected in leaf length. Leaves, therefore, should be a primary indicator of stress from toxic agents in Vallisneria. Under natural conditions, leaves of Vallisneria may reach 1.5 meters. Laboratory grown plants in the control microcosms produced leaves up to 1 meter in length. Leaf sloughing or breakage occurs in natural conditions, particularly where wave action is more intense. Leaf sloughing also occurred in laboratory grown plants. This phenomenon does complicate leaf length studies since only intact leaves may be measured after periods of culture.

Table 5 compares the number of intact leaves, total length and average length of leaves from control, and Atrazine dosed microcosms at the end of each of the three experimental periods in 1983. If the number of leaves in the first experimental period is examined, it is seen that the control microcosms produced approximately 50% more intact

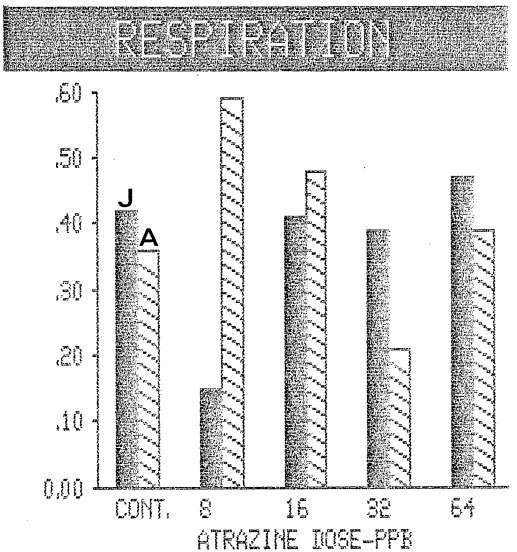


Fig. 4. Average oxygen consumption (microliters/½ hr) for control and Atrazine dosed flasks for the June and August 1983 experimental periods.

TABLE 5

COMPARISON OF NUMBER OF INTACT LEAVES, TOTAL LENGTH AND AVERAGE LENGTH OF LEAVES PER MICROCOSM FROM CONTROL AND DOSED MICROCOSMS AT THE END OF EACH OF THE THREE EXPERIMENTAL PERIODS, 1983

Atrazine Dose (ppb)	Number of Intact Leaves			Total Intact Leaf Length (cm)			Average Intact Leaf Length (cm)		
	Experimental Period								
	A	B	C	A	B	C	A	B	C
Control	160	100	118	3,915	4,604	3,823	24.5	47.5	32.4
*4	103	78	-	1,765	4,016	-	17.1	52.8	-
8	109	91	108	1,709	4,862	3,605	15.7	53.4	34.4
16	118	79	67	2,465	3,307	1,903	20.9	41.9	28.4
32	114	53	104	2,437	1,388	3,041	21.4	26.2	29.2
64	108	40	98	2,268	1,401	2,838	21.0	35.0	28.9

*Incomplete data for segment C.

leaves than the dosed microcosms which all produced about the same number of leaves. Conversely, in the second experimental period, a more linear decline is seen. The number of leaves in experimental period three does not seem to follow the pattern of the other two.

Average leaf length of the intact leaves during the first experimental period are relatively the same, with the control showing slightly longer leaves. However, the second and third experimental periods exhibit a sharp decline in leaf length at doses of 16 ppb and above. The decline is more notable in the second than in the third period.

Total leaf length for the dosed microcosms in the first experimental period ranged from 1700 cm to 2500 cm. However, the control had 63 to 129% more total length than all of the dosed microcosms. The pattern of decline can be seen in figure 5, which plots total leaf length of all plants in the microcosms against Atrazine dose for all three growth phase periods. A binomially smoothed curve shows that there is a general decline in total leaf length, the most susceptible being growth period B at all dose levels. This growth period is characterized by continued leaf extension and production of new leaves and plants in natural conditions.

Another way of treating population characteristics is to lump data into frequency classes. Table 6 shows the total leaf length of each plant in each microcosm for the three growth periods. The data are pictorally presented in figures 6, 7, and 8, which show the frequency class data as a series of bar graphs. Three groups of plants (Control, 16 ppb and 64 ppb) are plotted against leaf length class. Results of

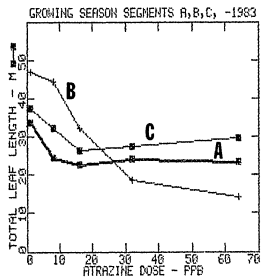


Fig. 5. Total leaf length of all plants in microcosms related to Atrazine dose for Segment A, B, and C growing periods.

TABLE 6

COMPARISON OF THE NUMBER OF PLANTS IN EACH MICROCOSM FALLING INTO EACH OF THE TOTAL LEAF LENGTH FREQUENCY CLASSES FOR EACH OF THE THREE EXPERIMENTAL PERIODS, 1983

Atrazine Dose	Frequency Distribution Classes														
	0-1.00m			1.01-2.00m			2.01-3.00m			3.01-4.00m			4.01-5.00m		
	Experimental Growth Period														
	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C
Control	15	3	2	9	6	3	2	5	8	4	4	4	0	2	0
8	26	2	2	4	4	6	0	7	9	0	5	1	0	1	0
16	18	10	7	8	4	9	1	3	0	1	2	1	0	2	0
32	12	15	7	10	4	8	2	1	3	0	0	2	0	0	0
64	17	10	8	5	4	8	2	0	4	1	0	1	0	0	0

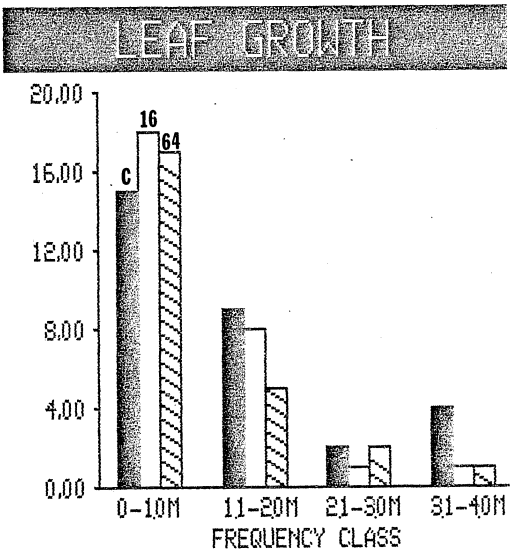


Fig. 6. Number of plants (Y-axis) with total leaf lengths that may be grouped into each of the four frequency classes, related to the control, 16 and 64 ppb Atrazine dosed microcosms. Growing season segment number A.

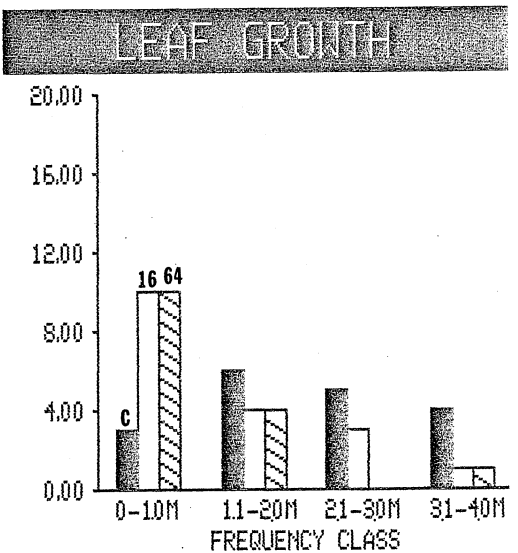


Fig. 7. Number of plants (Y-axis) with total leaf lengths that may be grouped into each of the four frequency classes, related to the control, 16 and 64 ppb Atrazine dosed microcosms. Growing season segment B.

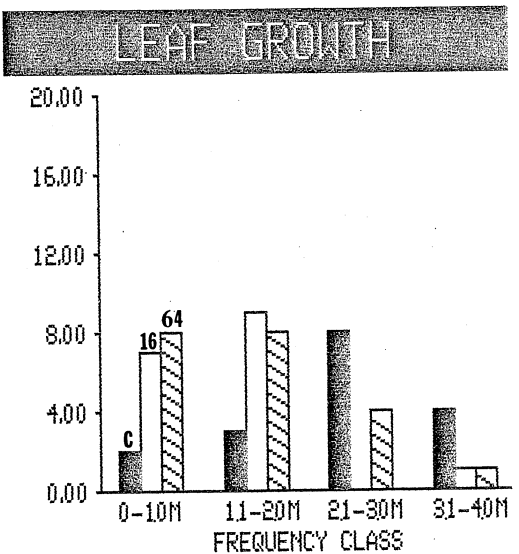


Fig. 8. Number of plants (Y-axis) with total leaf lengths that may be grouped into each of the four frequency classes, related to the control, 16 and 64 ppb microcosms. Growing season segment C.

frequency class data will be treated individually for each growth period.

Growth period A: May to mid-June. The results of growth period A in figure 6 show that the leaf length frequency in the control microcosms has a distribution which is skewed to the left from a normal distributon. This same type of skew to the left (with plants having shorter leaf lengths predominating) was obtained from the dosed microcosms, and even exceeded the number of short plants obtained from the control microcosm. Additionally, leaf extension in the class 3.01-4.00 m was depressed compared to the control.

Growth period B: mid-June to August. Results of growth period B (figure 7) show basically a normal distribution curve for the control microcosm with some new leaves and longer mature leaves, but with the mid-length classes predominating. However, the Atrazine dosed microcosms show a distribution curve with a skew to the left much like growth period A, with shorter plants predominating. Additionally, no plants within the longer classes exist in the 64 ppb microcosms.

Growth period C: August through September. Results of growth period C in the microcosms is shown in figure 8. A frequency class curve skewed to the right in the control microcosms is seen. The majority of the plants appear in the class of 2.10-3.00 m, which has longer mature leaves. The group that predominates next is that containing the longest leaves. However, plants grown in microcosms dosed with Atrazine continue to show a skew to the left, with short plants predominating. There were no plants from the 16 ppb microcosms falling into the class 2.10-3.00 m, and only one plant in the dosed microcosm in the longest leaf length class.

Biomass production

Whole-plant wet biomass (leaves, roots, and rhizomes) for all plants in each microcosm was measured prior to the beginning and at the end of the experimental period. Percent change in biomass was also computed.

Table 7 shows the data from a preliminary study performed in 1982. Experimental period B (7-2 to 8-3 1982) exhibits a severe reduction in biomass gain measured as percent change. The highest dosed microcosm, 64 ppb, showed no gain in biomass. At the end of experimental period C (8-4 to 9-15 1982), in which the plants are farther along in their life cycle, total biomass gain was depressed compared to the control microcosm but the downward trend was not quite as pronounced except at 64 ppb. As the concentration of Atrazine increased, the percent change in biomass decreased.

Table 8 reflects the data for the three experimental periods in 1983. It should be noted that beginning and ending data are slightly different for 1983 as compared to 1982. This adjustment will be treated in the discussion chapter.

The percent change in biomass that occurred in experimental period A of 1983 shows nearly a 55% decrease in net gain from the control to the microcosm dosed at 4 ppb. The decrease continues through the 16 ppb and then takes a slight increase. Figure 9 graphs the percent change for these microcosms. Slight increases that were observed at the 32 and 64 ppb never exceeded the depression observed for the 8 ppb dosed microcosm.

The decrease in percent change that was seen in experimental period A is also observed in period B except after a drop of 35% from

TABLE 7

BIOMASS CHANGE FOR EACH OF THREE GROWTH PERIODS IN 1982 SHOWING BEGINNING (B), ENDING (E), AND PERCENT CHANGE (C)

	Biomass (Gms)								
Atrazine	5/21 to 7/2/82			7/2 to 8/3/82			8/4/ to 9/15/82		
Dose	B	E	C%	B	E	C%	B	E	C%
Control	20.7	39.7	111	-	-	-	70.4	131.8	87.5
4	-	-	-	-	-	-	39.5	71.8	81.5
8	-	-	-	-	-	-	53.9	80.1	48.6
16	-	-	-	45.4	67.2	48	74.1	131.6	77.6
32	-	-	-	47.0	58.6	24	70.2	118.5	68.8
64	-	-	-	28.2	28.1	0	66.7	84.1	26.0

TABLE 8

BIOMASS CHANGE FOR EACH OF THE THREE GROWTH PERIODS IN 1983, SHOWING BEGINNING (B), ENDING (E), AND PERCENT CHANGE (C)

	Biomass (Gms)								
	A			B			C		
Atrazine	5/26 to 6/23/83			7/21 to 8/17/83			8/23 to 10/6/83		
Dose	B	E	C%	B	E	C%	B	E	C%
Control	33.8	82.9	145.3	59.8	120.2	101.0	93.6	145.5	55.4
4	30.4	50.1	64.8	67.1	111.6	66.3	*	*	*
8	31.5	46.9	48.9	82.1	125.7	53.1	91.8	137.9	50.2
16	37.0	48.0	29.7	78.3	105.5	34.7	90.8	109.2	20.3
32	32.1	44.2	37.7	80.1	110.6	38.1	100.2	116.7	16.5
64	32.5	48.2	48.3	81.2	89.2	10.2	99.3	106.6	7.4

*Microcosm damaged.

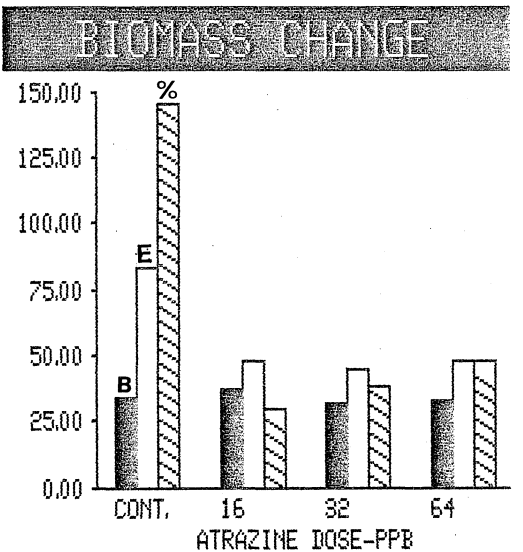


Fig. 9. Wet-weight biomass in grams (Y-axis) for the beginning (B), the end (E), and percent change (%) for the control and Atrazine dosed microcosms. Growing season segment A, 1983.

the control, which doubled its biomass, a steady decrease is observed. This is similar to the 1982 data from period B. A bar graph demonstrating this decline is seen in figure 10.

Experimental period C also had a decline in biomass gain, but the dramatic decrease was not seen until the 16 ppb and higher dosed microcosms. This pronounced drop is displayed in figure 11. Here the 16 ppb microcosm has a net gain of less than one-half that of the control.

Reproductive Capacity: Rhizomes; Male and Female Flowers; Tubers

The immediate success and long-term survival of a species is dependent upon production of new individuals. In Vallisneria this occurs in three basic ways: production of new individuals during a growing season via horizontal rhizomes from parent plants, sexual reproduction resulting in seeds which over-winter and germinate near the parent plants or are transported for establishment of new beds, and production of tubers via vertical rhizomes which over-winter and produce new individuals for a new growing season. Tables 9 and 10 show rhizome, flower, and tuber production in control and dosed microcosms for growing segment C in 1982 and 1983, respectively.

Reproduction from horizontal rhizomes

In natural conditions Vallisneria reproduces vegetatively in a profuse manner. One or more rhizomes from a parent plant is common, and during early to mid-season growth, as many as six individuals have been found attached as a result of rhizome extension from each new plant (personal observation). Horizontal rhizome production also occurred

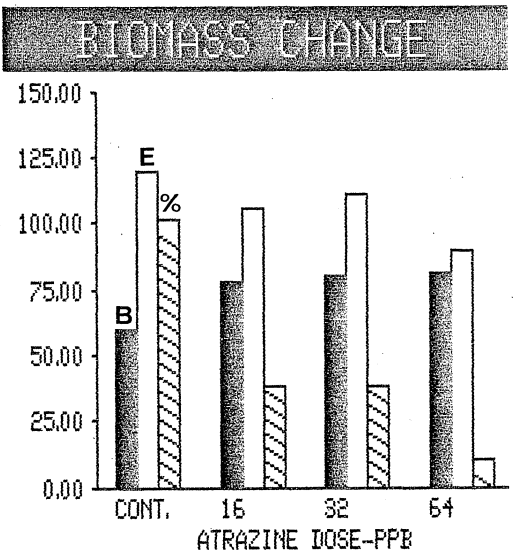


Fig. 10. Wet-weight biomass in grams (Y-axis) for the control and Atrazine dosed microcosms. Growing season segment B, 1983.

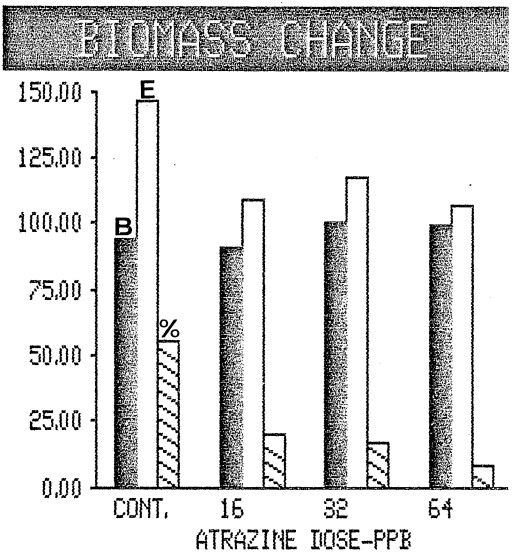


Fig. 11. Wet-weight biomass in grams (Y-axis) for the beginning (B), the end (E), and percent change (%) for the control and Atrazine dosed microcosms. Growing season segment C, 1983.

TABLE 9

EFFECTS OF VARYING CONCENTRATIONS OF ATRAZINE ON VEGETATIVE AND SEXUAL REPRODUCTION OF V. AMERICANA OCCURRING BETWEEN AUGUST 4, TO SEPTEMBER 15, 1982 (SEGMENT C)

Atrazine	No. Plants		Percent	Tubers		No. Flowers		Rhizome
ppb	Initial	Final	Change	Number	Percent of Control	Male	Female	Number
0	18	24	33.3	44		0	6	45
4	18	25	38.9	37	84.1	0	1	36
8	18	27	50.0	35	79.5	0	1	32
16	18	23	27.8	18	40.9	2	4	34
32	18	29	61.1	15	34.1	0	0	41
64	18	21	16.7	11	25.0	0	0	22*

*Rhizomes were stunted as compared with those from other microcosms.

TABLE 10

EFFECTS OF VARYING CONCENTRATIONS OF ATRAZINE ON VEGETATIVE AND SEXUAL REPRODUCTION OF V. AMERICANA OCCURRING BETWEEN AUGUST 23 TO OCTOBER 6, 1983 (SEGMENT C)

Atrazine	No. Plants		Percent	Tubers		No. Flowers		Rhizome
ppb	Initial	Final	Change	Number	Percent of Control	Male	Female	Number
0	18	18	0	72	-	7	5	19
8	18	18	0	74	102.8	18	6	15
16	18	18	0	60	83.3	10	0	13
32	18	20	11.1	57	79.2	12**	0	16
64	18	21	16.7	40	55.6	4**	0	8*

*Rhizomes small and fragile.

**Flowers stunted and discolored.

Note: Flowering and rhizome data collected between July 21, 1983 and August 17, 1983.

commonly in microcosm grown specimens of Vallisneria. The number of individuals doubled during an experimental run, in some cases.

If the number of rhizomes produced in each microcosm is examined in table 9, the range is between 32 and 45 in microcosms dosed at 32 ppb and lower. At 64 ppb the number was decreased by a factor of 2 when compared to the control. Additionally, the gross morphology of the rhizome was altered compared to the controls. The rhizomes were fragile and appeared quite stunted.

The rhizome data for 1983 is shown in table 10. Again, at 64 ppb the gross morphology of the plant was altered. The rhizomes were small, fragile, and somewhat decomposed. Again, the total number of rhizomes produced was more than halved at the highest dose.

Sexual reproduction from male and female flowers

In field conditions, Vallisneria flowers profusely produce male and female flowers on separate plants. Female plants appear to be more common than male plants. The male flower is produced on a short (about 5-8 cm) stalk, while the female flower extends to the surface of the water on long (up to 1 meter) peduncles.

In the control microcosms, both male and female flowers were produced. Table 9 illustrates data collected during the 1982 growing season for both control and Atrazine dosed microcosms. The number of female flowers produced was not significantly correlated with Atrazine dose although none were recorded above 16 ppb. There were fewer male flowers produced in all microcosms which probably reflects the sex of the plants collected rather than dose.

During 1983 (table 10), no female flowers were observed in microcosms dosed with Atrazine above 8 ppb. Male flowers that were produced followed no observable pattern, but at the highest dosages of Atrazine, appeared quite discolored and were very stunted. None of the stalks exceeded 1 cm, compared to the control microcosms in which stalks exceeded 5 cm in length.

Reproduction from over-wintering tubers

Tuber production in the field occurs after production of a vertically grown rhizome, which extends from 10 to 20 cm into the substrate. Most individual plants will produce one or two of these special rhizomes.

Table 9 shows that the number of tubers produced in 1982 were reduced with increasing levels of Atrazine. At the lower concentrations of 4 and 8 ppb, tuber production was reduced by 16 and 20.5%, respectively. A dramatic drop is seen between the microcosms dosed at 8 ppb and 16 ppb. There the number is halved. The reduction continues as the dose of Atrazine is increased, finally reaching only 25% that of the control, or a 75% reduction at 64 ppb. This phenomenon was observed again during the 1983 experimental periods (table 10). Figure 12 is a bar graph illustrating the reduction in number with increased dose levels during 1983. A progressive decline in tuber number was seen especially in the microcosms dosed above 8 ppb. At 64 ppb the number of tubers was slightly more than half of the control.

Tuber biomass for each microcosm was also recorded. The weight of a given tuber was also reduced with increasing dose levels (indicating less photosynthate available for storage in the tuber). Figure 13

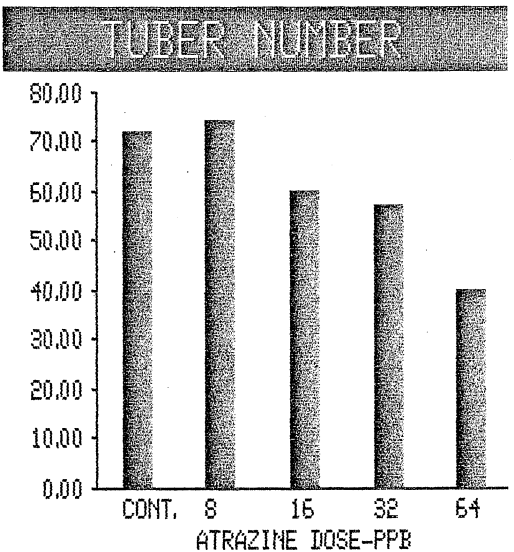


Fig. 12. The number of tubers (Y-axis) produced in the control microcosm (in 1983) compared to the number produced in each of the Atrazine dosed microcosms.

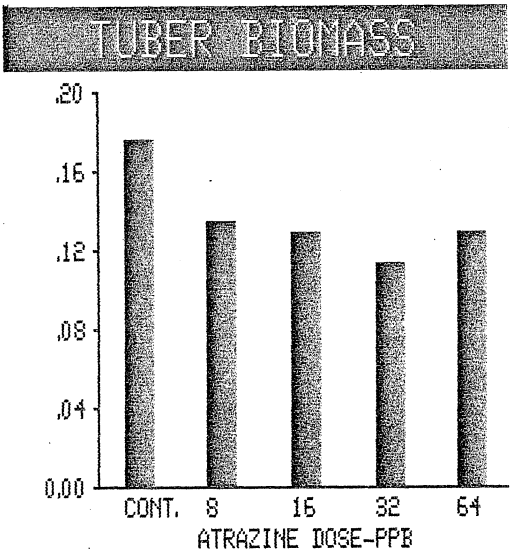


Fig. 13. Compares average biomass (grams per tuber) of tubers produced in the control and Atrazine dosed microcosms, 1983.

is a bar plot showing that at all doses at 8 ppb or above, there was a weight reduction in each tuber compared to control grown tubers.

CHAPTER VIII

DISCUSSION

A laboratory microcosm system has been designed which may be used for any commonly occurring submersed vascular plant. This system was successfully tested using Vallisneria americana as a test species. The first plant culturing experiments conducted without Atrazine dosing exhibited more than a 100% increase from the initial number of plants and had biomass gains ranging from 77.6 to 138.8%. No casualties were observed from the 72 plants that were gathered randomly from the field in May of 1982, and planted in the microcosms.

Other researchers have reported losses of control plants when V. americana was used as a test species. It is likely that certain optimum growing conditions were not met. Research has shown that maximum photosynthesis using the Gilson Respirometer, occurs in V. americana in a light regime of 270 $\mu E\ m^{-2}\ sec^{-1}$ and that photosynthesis is decreased at both higher and lower light levels (Anderson, unpublished data).

The pH level also affects photosynthesis in V. americana. Carbon uptake is decreased when pH is increased from 7 to 8 (Titus, 1980). Some researchers have conducted their experiments at pH levels of 7 plus and this might account for lack of optimum growth of controls and/or for the mortality rate reported (Correll, 1982; Forney & Davis, 1981).

Salinity, too, can be a factor in successful cultivation. V. americana thrives in fresh to slightly brackish waters of less than 3 ppt (Haller et al., 1974). A salinity of 1-2 ppt was chosen for the microcosms since that most closely represented field conditions (personal observation). Although V. americana was recorded growing in waters of 10 ppt during the summer of 1981 (personal observation), this salinity was reached gradually beginning in the spring and peaked in August. This slow acclimation is quite different from gathering plants in low salinity conditions and then placing them immediately in higher salinities. Lack of acclimation and high salinities (5 and 8 ppt) may account for the 50% mortality that Correll (1982) reported, when V. americana was exposed to 12 ppb of Atrazine for 47 days. It is unclear if this was the case.

Temperature of the microcosms should also be kept constant (Haller, 1974). A temperature of 25 C more closely approximates field conditions during the growth phase. The 20 C used by Haller (1974) is somewhat low for optimum growth. Under field conditions in bodies of water such as the collecting sites (Port Tobacco River) temperature at the level of the base of the beds does not fluctuate more than 1 C (personal observation). A day-time/night-time temperature regime of 26 C/15 C, respectively (as well as the light levels) used by Forney and Davis (1981) could account for the low growth of their controls.

Circulation around the bed which distributes CO_2 and O_2 throughout the system is crucial to optimum growth. Other research has been conducted in a single aquarium, which would not allow for circulation.

It appears to be vitally important for optimum growth in control microcosms to regulate physical parameters such as light, temperature, pH, and salinity so that no fluctuation occurs. Ideal field conditions should be simulated in microcosms, so that physical parameters do not become a confounding factor.

All of these parameters were extensively studied and incorporated into the design of the microcosm system in this research. In all experimental periods encompassing two entire growing seasons of 1982 and 1983, respectively, optimum change in biomass as well as all phenophases of the life cycle which occur in the natural habitat of Vallisneria were duplicated. Mortality of Vallisneria plants was never encountered in this research. It is these results that attest to the success of the design.

Effect of Various Concentrations of Atrazine on Metabolic Rates, Growth, and Reproduction

Effect of Various Concentrations of Atrazine on Photosynthetic and Respiratory Rates

Photosynthesis

Short-term experiments using the Gilson Respirometer seem to indicate a slight stimulation of photosynthesis at all doses of Atrazine except 64 ppb. The 8 ppb is the most affected. Stimulation of photosynthesis has been reported by other researchers working with the triazines (Ashton et al., 1960; Beaumont et al., 1980; Roth, 1958). When the bar plot of oxygen evolved versus dose is examined (figure 2), very little suppression as compared to the control rate is seen.

However, the control flasks do become CO_2 limited and hence do not show a true rate of photosynthesis. In fact, tests showed that at the end of 4 hours no CO_2 was present in any flask.

When oxygen production (in $\mu\ell$) was plotted against time (figure 3), a peak for all doses was reached within a half hour of closure to the atmosphere. The only exception to this was for the 64 ppb dose. Here the peak was not reached until one hour had elapsed. This could be an indication of suppression of photosynthesis.

After this peak is reached all dosed flasks exhibit a decline. The reason for this decline is twofold. One factor is the gradual reduction in CO_2 availability, which eventually becomes the limiting factor approximately three hours after beginning the experiment. The other factor is the penetration of Atrazine into the chloroplastid of the leaf cells and an ultimate reduction in photosynthetic capacity.

Separating the effect of CO_2 limitation from that of Atrazine dose may be difficult; however, it is interesting to note that the curves of the dosed flasks show a lag in the reduction of oxygen evolution compared to the control flasks (figure 3). One interpretation is that Atrazine is limiting photosynthetic activity and hence CO_2 does not become a limiting factor as rapidly as in the control flasks.

However, a need exists to conduct further research to reduce or eliminate the variable of CO_2 limitation in order to better understand time interval of Atrazine penetration into leaf cells, and photosynthetic suppression on a short-term basis.

Respiration

There appears to be no clear-cut relationship between Atrazine dose and oxygen consumption, although small concentrations (8 ppb) in June did result in significant reduction in consumption (figure 4). The August data showed the opposite effect. Oxygen consumption of 70% or more than that of the control was seen. Other research has shown that triazines at low doses caused a stimulation in respiration (Ashton et al., 1960; Roth, 1958). Doses of 8 ppb and 16 ppb in August agree with this finding.

Effect of Various Concentrations of Atrazine on Leaf Growth and Biomass Production

Leaf Growth

If total leaf number (both broken and intact leaves) is compared to number of intact leaves (table 5), it is seen that the same pattern occurs for each experimental period. Figure 14 which is a bar plot of total number of leaves in each experimental period of 1983, illustrates the suppression of growth that occurs in every dosed tank for the first growth period. This suppression is not as dramatic in the second growth period and only occurs at the highest dose.

This pattern could be due to the greater susceptibility of the younger plants very early in their life cycle. When the plants are more mature less and less of an effect on leaf number seems to occur. This is especially evident in the last growth period in which little difference in leaf number is seen in all dosed microcosms.

Figure 15, a binomial smoothed curve, illustrates this suppression in the early growth cycle, less so during the summer months

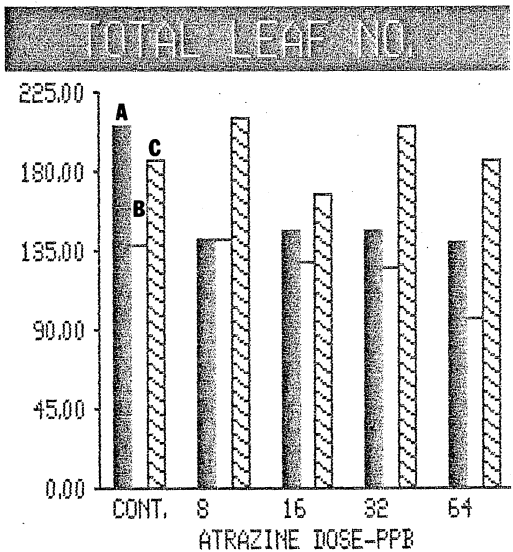


Fig. 14. Total number of leaves produced in control and Atrazine dosed microcosms for the three growing season segments, 1983.

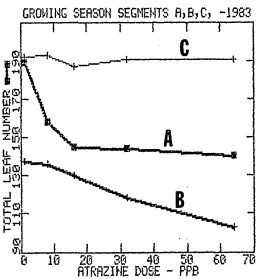


Fig. 15. Compares suppression of leaf growth by Atrazine in each of the three growing season segments, 1983.

(segment B) and finally little suppression as the plants complete sexual reproduction and begin tuber production (segment C).

It becomes apparent that if an ephemeral pulse of Atrazine occurs early in the growth cycle (for example in May which is the time that Atrazine is first applied to corn fields), total leaf number would be affected. This, in turn, could have indirect consequences of reduction in total leaf surface for photosynthesis. Reduction in photosynthesis could then affect tuber formation which is the prime means that the plant has of over-wintering.

Average leaf length

When the average length of intact leaves is examined, as shown in figure 16, it can be seen that there is a decrease in length during the second and third experimental periods at doses of 8 ppb and above. If this information is pooled with that of total leaf number, it appears that although in the third growth phase the number of leaves are relatively the same, the average leaf length is less. This is even more pronounced in the second growth period at doses above 8 ppb.

This means that the plants might have the same number of leaves, but that the leaves are shorter and overall surface area for photosynthesis would be decreased. Therefore, ephemeral pulses above 8 ppb occurring in late June and July could affect subsequent carbon fixation needed by the plant for flowering and tuber production.

Total leaf length

Total leaf length included the intact leaves only and did not include the length of a leaf to its broken edge since it was impossible to determine the total length of a leaf prior to sloughing. Sloughing

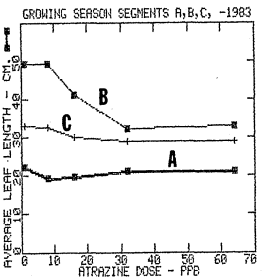


Fig. 16. Compares average length of leaves related to Atrazine concentration in each of the three growing season segments, 1983.

is a natural phenomenon and can be intensified by wave action. However, in the microcosms the variable of waves was eliminated and only breaking due to aging or other causes could be observed. It appeared, however, that Atrazine intensified this occurrence during the second growth phase but less so during the third growth period. This is illustrated in figure 17. The second growth phase shows a nearly linear increase in percentage of broken leaves as dose is increased.

At higher doses the leaves of the plants folded and broke before chlorosis was even noted. Again, if Atrazine compounds the sloughing phenomenon, then fewer intact leaves would be left. Figure 18, which again examines sloughing, plots the number of intact leaves for all three growth phases, for each dose. Segment B has the fewest intact leaves. Therefore, when total leaf length of intact leaves is analyzed, sloughing effects need to be considered. The more sloughing that occurs, fewer intact leaves will prevail, and this will be reflected in total length.

The regression of total leaf length and dose of Atrazine (figure 19) demonstrates that the leaf length decreased as the dose increased during segment A. The negative slope (-8.28) is indicative of a downward trend, and further supported by a correlation of -0.70. Sloughing does not appear to be a factor in this downward trend in the first growth period.

Figure 20 is the regression for segment B. Here the effect of sloughing is dramatically portrayed by the steep negative slope of -20.13. The correlation of -0.83 indicates that Atrazine appears to inversely affect the total leaf length.

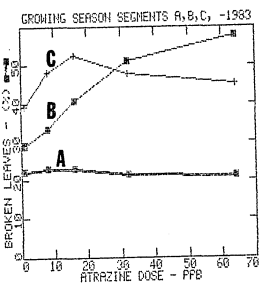


Fig. 17. Compares number of broken leaves with that of the control to various Atrazine doses.

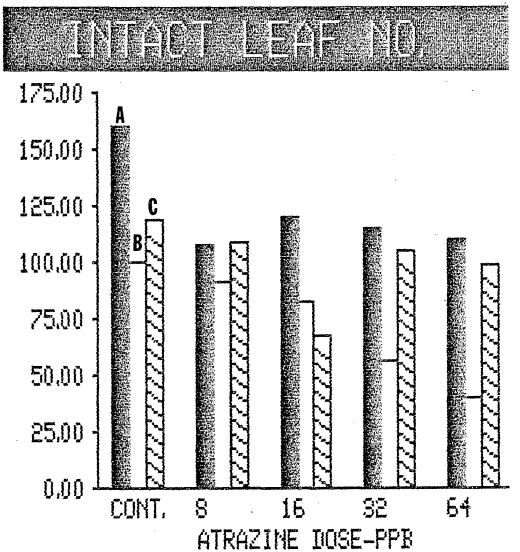


Fig. 18. Compares number of intact leaves for the control and Atrazine dosed microcosms in each of the three growing season segments, 1983.

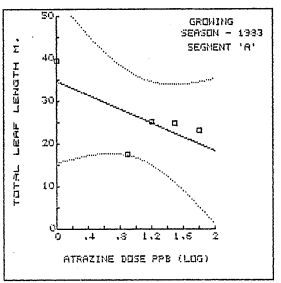


Fig. 19. Regression plot of total leaf length against Atrazine dose-growing season segment "A", 1983.

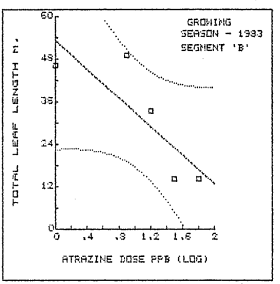


Fig. 20. Regression plot of total leaf length against Atrazine dose-growing season segment "B", 1983.

A negative slope -6.40 is again seen for the last growth phase (figure 21), very similar to but less than that of the first growth period, with a correlation of -0.59.

From the regression curves it can be surmised that an ephemeral dose of Atrazine occurring at the beginning of segment A or C would appear to cause some decrease in total leaf length of intact leaves of Vallisneria.

The growing period that is characterized by continued leaf extension and the beginning of sexual reproduction, segment B, is the most notably affected by an ephemeral dose of Atrazine. This regression line had the steepest slope and highest correlation of the three. Whether Atrazine operates by increased sloughing or decreasing the length of an intact leaf, the effect is the same, less surface area to be illuminated, which directly affects maximum photosynthesis. The likelihood of this ephemeral pulse occurring during the summer months is very great when environmental conditions are considered. Sudden summer thundershowers contribute to washoff of chemicals applied to surrounding farmlands and heavy sediment loads in the tributaries. The summer of 1984 brought just such conditions to the Maryland tributaries of the Chesapeake Bay where rivers were silt laden and deep ruts of erosion could be seen in farmlands (personal observation).

Frequency classes

Sampling of Vallisneria beds in natural conditions and grouping according to leaf length frequency during growth segment A would show a skew of the normal distribution curve to the left with plants having shorter leaf lengths predominating. Results of microcosm growth period

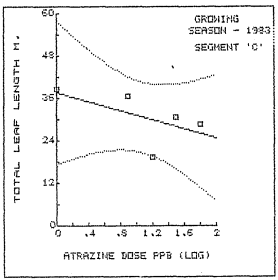


Fig. 21. Regression plot of total leaf length against Atrazine dose-growing season segment "C", 1983.

A show that the expected leaf length frequency was obtained in the control microcosms. The same type of skew was obtained from the dosed microcosms. The fact that slightly more plants having total leaf lengths less than 1.00 m were recorded for doses of 16 ppb and above as compared to the control (figure 6), could indicate that growth was inhibited allowing plants with shorter leaves to predominate. This conclusion is further substantiated by the suppression in number of plants occurring in the longest leaf class.

Growth segment B is normally a time of leaf growth and maturation in natural conditions. The frequency classes would be expected to approach a normal distribution. This was, indeed, the finding in the control microcosm, but not in microcosms above 8 ppb (figure 7). The pronounced skewness to the left for doses above 8 ppb indicates that growth is curtailed, and shorter plants prevail. This, again, would substantiate the finding of a steep negative slope of the regression line. In fact, virtually no plants were recorded that had total leaf length above 2.00 m for doses above 16 ppb.

The third growth segment (C) is a period of lessened new leaf production and maturation of leaves. Under normal growth conditions one would expect to find the leaf growth frequency curve to be skewed to the right with mid- and longer leaf lengths predominating. This pattern was observed in the control tanks, but not in the dosed microcosm above 8 ppb (figure 8). The dosed tanks above 8 ppb exhibited a skewness to the left indicating an inhibition of leaf growth. The 8 ppb microcosm, which more approaches the control microcosms, had a notable absence of plants whose total leaf length was above 3.00 m. The possibility, therefore, of a subtle sublethal stress even at this low dose exists.

Biomass Production

Growth segments A and B were purposefully chosen to be 4 weeks in length, while segment C was allowed to continue for 6 weeks. Initial experiments performed during the growing season in 1981 and 1982 indicated that a percentage change well over 100% could be obtained in control microcosms during that time. However, tuber formation spans a longer time period, hence the additional time.

When comparing one segment to another it should be kept in mind that an absolute comparison cannot be made, but the intent from the beginning was to examine trends. Moreover the selection of a date to begin the experimental period also affects the absolute percentage change that occurs. For example, segment C in 1983 was begun 3 weeks later than in 1982. This, however, does not shadow any trend, in fact it sheds light as to what effect an ephemeral pulse would have if it occurred in the field at different times.

The first dosing experiments were begun during segment B of 1982. Since sublethal effects of low dosages of Atrazine, simulating an ephemeral pulse were of prime concern, the values of 16, 32, and 64 ppb were chosen. The trend was a very definitive downward one with the lowest dose of 16 ppb resulting in a 48% change in biomass.

The dosage regime for the last segment of 1982 was, therefore, extended to even lower doses to further explore sublethal effects. Again, the downward trend is graphically portrayed in figure 22. When biomass as percent change is regressed against the log of the dose (figure 23) a slope of -24.2 is found with a correlation of -0.68. There appeared to be a definite inverse relationship between biomass and Atrazine dose during this segment. This is the time of flower and tuber

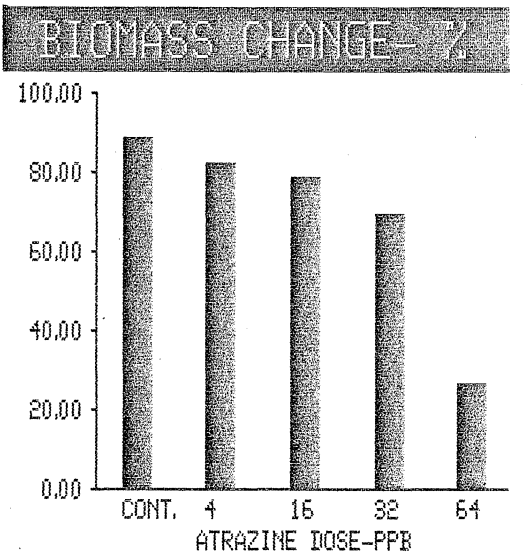


Fig. 22. Biomass change related to Atrazine dose-growing season segment C, 1982.

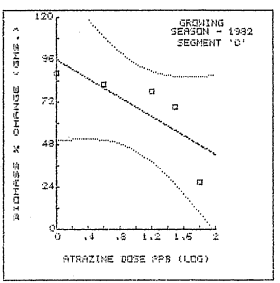


Fig. 23. Regression plot of percent biomass change vs. Atrazine dose-growing season segment "C", 1982.

production, so most of the biomass gain is not a result of leaf extension, as the leaf data of 1982 and 1983 demonstrate.

The three growth segments percentage of biomass for 1983 are illustrated in figure 24. In all growth periods the greatest percent gain in whole-plant biomass occurred in the undosed, control microcosms. All dosed microcosms showed significant reductions in biomass as compared to the controls. The most dramatic suppression of growth as shown in figure 25, a binomial smoothed curve, occurred at the lowest doses, namely, those at 16 ppb and below.

When regression lines of the individual growth segments were examined (figures 26a, b, and c), all showed negative slopes and high negative correlations. Segment A had the greatest negative slope, -53.5 and a correlation of -0.82. A similar negative slope of -46.9 was found for segment B, but with extremely high correlation of -0.98. The pattern was again followed in segment C with a slope of -28.3 and a correlation of -0.91. For comparison purposes, these three regression lines are overlayed in figure 27. Here it can be seen that segment A is the most affected from a single ephemeral pulse of Atrazine when total biomass is considered. This is precisely the time when preemergent applicaiton is taking place, and the first ephemeral pulse might occur.

It is also interesting to note how all three regression lines appear to merge toward 0% change at doses of 64 ppb. This could be interpreted as a point beyond which sublethal effects cease and direct mortality occurs.

There appeared to be a need to separate the biomass of the tubers out of the total biomass content for segment C. This was done and is discussed in the section on tuber production.

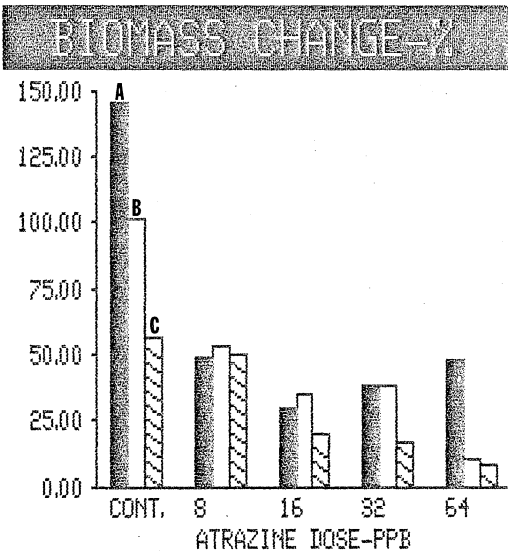


Fig. 24. Biomass change related to Atrazine dose-growing season segments A, B, and C, 1983.

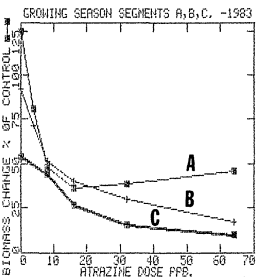
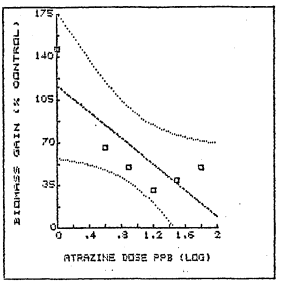
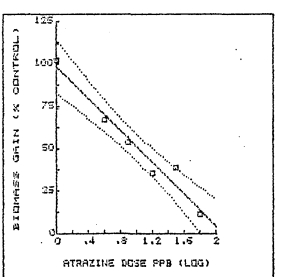


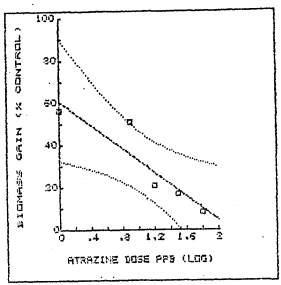
Fig. 25. Compares percent biomass change with Atrazine dose-growing season segments A, B, and C, 1983.



A



B



C

Fig. 26. Regression plot of percent biomass gain vs. Atrazine dose-growing season, segments A, B, and C, 1983.

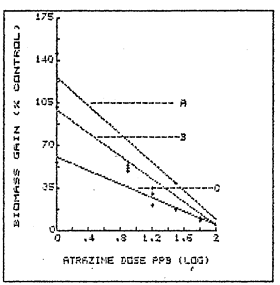


Fig. 27. Overlay of three regression plots as shown separately in figure 26 A, B, and C, growing season segments A, B, and C, 1983.

Reproductive Capacity

Vegetative reproduction from rhizomes

Data from both 1982 and 1983 are similar. Although the number of rhizomes were halved at 64 ppb and the gross morphology was altered, a regression curve, as shown in figure 28, demonstrates a general downward trend in numbers of rhizomes as the dose of Atrazine is increased. A correlation of -0.82 was calculated for 1983. The number of rhizomes, therefore, may be affected by a low ephemeral pulse. It seems apparent that concentrations above 32 ppb would begin to seriously affect rhizome production.

Sexual reproduction from male and female flowers

The 1982 data collected from microcosms did not follow a definitive trend for female flowers at low doses. The number produced was more likely correlated with random selection of the plants, since male and female flowers are produced on separate plants. However, at doses of 32 ppb and higher no flowering occurred, and this phenomenon was repeated during 1983. In fact, in 1983, the data indicated that flowering of female plants is repressed at doses of 16 ppb and higher.

In 1983, although numbers of male flowers did not seem to follow any pattern, the morphology was severely affected at doses of 32 ppb and 64 ppb. Since the male flower appeared very stunted (all heights were less than 1 cm), it would be interesting to conduct further research on the viability of the pollen. Since it was observed during the course of this research that seeds contribute to the extension of beds and to colonizing new areas, ephemeral doses of Atrazine at the higher concen-

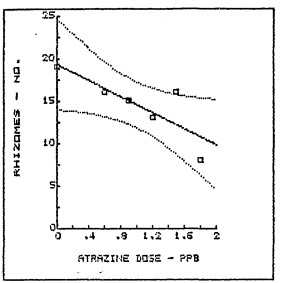


Fig. 28. Regression plot of rhizome number vs. Atrazine dose, 1983.

trations tested could affect successful reproduction if the seed is not viable. The heads of tributaries are often the location of the greatest ephemeral pulses, being directly adjacent to farmland washoff. This is precisely the place where new colonies often begin, when seed is carried in with the tide.

Reproduction from over-wintering tubers

The survival of Vallisneria beds in a given locality appears mostly dependent on the production of new plants from over-wintering tubers at the beginning of a new growing season. Although seeds do germinate, this is not thought to be a primary factor in bed survival (Gleason, 1974). The most significant results of this research on the sublethal effects of Atrazine came from those related to tuber production.

Figure 29 depicts the downward trend in tuber production for 1982. Even at the lowest doses tested (4 and 8 ppb), production is 16% and 21% reduced, respectively. When the dose is increased to 16 ppb, tuber production was suppressed nearly 60%.

The 1983 data confirm the findings in 1982. Figure 30 shows this sudden downward trend at doses above 8 ppb. Furthermore, when the regression line for 1982 and 1983 is examined in figures 31 and 32, tuber production has steep negative slopes of -20.1 and -16.3, respectively. The very high negative correlations of -0.96 and -0.97, respectively, for the two years illustrate the substantial sublethal effect that Atrazine has on tuber production. The effect is most pronounced at doses above 8 ppb.

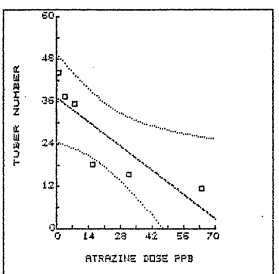


Fig. 29. Regression plot of tuber number vs. Atrazine dose, 1982.

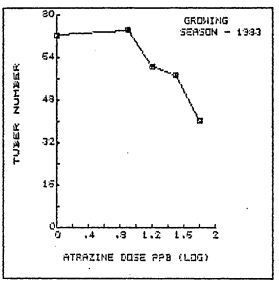


Fig. 30. Semi-log plot of tuber number vs. Atrazine dose, 1983.

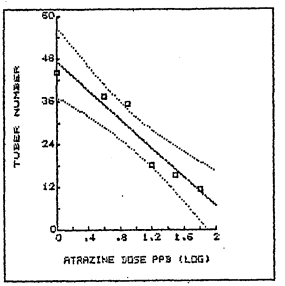


Fig. 31. Regression plot of tuber number vs. Atrazine dose, 1982.

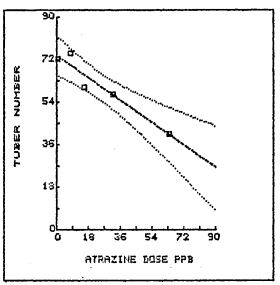


Fig. 32. Regression plot of tuber number vs. Atrazine dose, 1983.

In addition to an effect on tuber number, the biomass of the tubers that were produced showed a downward trend. Figure 33 which plots tuber biomass in 1983 versus the log of Atrazine dose demonstrates this decrease in biomass from the control. There is an immediate decrease at all doses tested. When a regression line is drawn, as in figure 34, a negative slope of -30.6 indicates a reduction in stored food. Furthermore, a correlation of -0.90 represents a strong indication of decreased biomass of the tubers at all doses. These findings were quite evident when the gross morphology of the tubers was examined. Especially at the highest doses tested, the V-shaped tuber was quite small, compared to the controls.

How serious this reduction is in stored food to a given tuber producing a new plant at the beginning of a growing season, remains to be researched. If there is insufficient food reserve to maintain the tuber through the winter months and then extend a new plant to the substrate surface the following spring, bed survival may be endangered.

The implications of these findings on tuber production are pronounced. If one single ephemeral pulse can more than halve the total number of tubers, it can be readily seen that in a few years extensive beds of Vallisneria could be substantially reduced, if not eliminated. Even if the tubers are produced, the fact that they are small and contain less stored food could further reduce the likelihood that they would produce a healthy plant the following spring.

Moreover, it was found that leaf length was most affected during the second phase of growth (segment B). This reduction in potential photosynthetic surface area could lead to further reduction in tuber number and biomass, if the ephemeral exposure occurred in July as

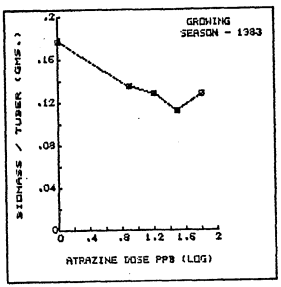


Fig. 33. Semi-log plot of tuber biomass vs. Atrazine dose, 1983.

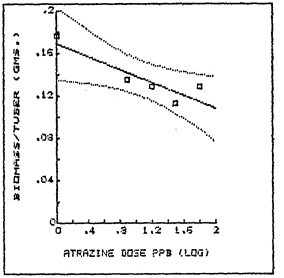


Fig. 34. Regression plot of biomass per tuber vs. Atrazine dose, 1983.

opposed to the third growth period, segment C. In this segment plants were farther along in their life cycle prior to exposure.

This suggestion is further substantiated by the difference in tuber number that occurred during 1982 and 1983. In each of the two years tuber number was significantly reduced although more total tubers were recorded in 1983. The exposure of the plants to Atrazine in 1983 occurred 19 days later than in 1982. It, therefore, appears to be very critical when (in the life cycle) exposure occurs. The earlier in August that exposure occurs, when vertical rhizoming has not begun, the more stressful it is in terms of resulting tuber production.

Adverse effects to reproduction and development, even when immediate survival was not affected, have been documented by many researchers for aquatic animals exposed to herbicides (Bowman et al., 1981; Macek et al., 1976; Schober & Lampert, 1977). Environmental studies, which considered either the particular phase of a plant's life cycle in which herbicide exposure occurred, or the sublethal effects on reproduction are nonexistent.

Finally, this research has considered the "least worse cases": a single ephemeral dose exposing Vallisneria plants. It is very likely that in natural conditions, with numerous rain showers and thunderstorms over the course of a growing season and repeated applications of Atrazine, Vallisneria beds would almost certainly have repeated exposures. This could compound the sublethal effect observed in this study, resulting in even more devastating effects to this vitally important species.

CHAPTER IX

CONCLUSIONS

The emphasis of this research was to develop a model, by which chemical toxicity effects on submersed vascular plants could be studied. It was felt that there was a twofold aspect to this approach:

1. To understand the physical and chemical behavior of the chemical in question, including application procedures, fate in the environment, and biological circumstances that would permit exposure to sensitive organisms.

2. To design a system by which the life history of an important aquatic plant exposed to a chemical could be studied.

Conclusions for this research were then drawn with respect to findings from the above. This was accomplished by examining the effects of Atrazine at low concentrations on the entire life cycle of Vallisneria americana.

1. Leaf growth appears to be affected at all times during the growing season at concentrations above 8 ppb.

2. Whole plant biomass is profoundly affected at concentrations above 8 ppb at all times during the growing season.

3. Sexual reproduction was affected at concentrations of 32 ppb and above in male flowers. Viability of pollen needs to be investigated.

4. Vegetative reproduction in the form of tubers was drastically affected. Concentrations exceeding 8 ppb not only decreased numbers, but reduced tuber weight.

It is, therefore, apparent that a critical problem exists in terms of concentrations of Atrazine that make their way into the water column and the concurrent loss of Vallisneria beds that have occurred in the Chesapeake Bay watershed. Atrazine after all is and was developed to be a powerful herbicide. It has been instrumental in increasing corn yields and reducing labor costs.

However, bed loss has coincided with accelerated erosion of farmland in this country. When costs of farming began to increase, farmers often opted to cut down on costly conservation practices such as crop rotation and terracing. Soil erosion is often more than 1 cm of top soil yearly (Miller, 1985). Most of this soil is being washed into the nation's waterways, frequently carrying with it adsorbed persistent herbicides like Atrazine. Misuse of this herbicide coupled with poor conservation has allowed ephemeral pulses of this herbicide to expose a submersed plant like V. americana (personal observation).

Furthermore, much of the research which has been done on the effects of Atrazine and other herbicides has been shortsighted, in that all phases of the life cycle of a given species such as Vallisneria have not been investigated for sublethal, but nonetheless devastating, effects. No mention is ever made in most studies as to what phase of growth the particular plant is in, no true understanding of the life history of these plants exists, and toxicity studies usually focus only on EC_{50}. This approach has led to recent statements dismissing herbi-

cides as a factor in the loss of submersed plant beds. The conclusions reached are not only premature but dangerous.

Studies such as this one should be expanded to include other herbicides and other important submersed aquatic macrophytes. Particular focus needs to be made on sublethal effects which can subtly deplete population numbers leading to the eventual elimination of plant beds by affecting their ability to over-winter.

APPENDIX

COMPUTER PROGRAM FOR THE GILSON RESPIROMETER

The following program was written to reduce data obtained from the Gilson Differential Respirometer. It was written for the TRS-80 Color Computer.

```
3 PRINT#-2, "GILSON"
5 INPUT "DATE OF GILSON RUN.DOSE OF HERBICIDE";A$
6 INPUT "TEMP C. LIGHT MICROEIN":B$
7 PRINT#-2,A$,B$
8 PRINT#-2, " "
9 PRINT#-2, " "
10 INPUT"# OF CONTROL FL":Y
20 INPUT"# OF RESPIR FL";X
30 INPUT"# OF EXPER FL":V
40 INPUT"# OF READINGS";Z
45 INPUT "CORRECTION FACTOR":CF
50 DIMC(Y.Z),R(X,Z),E(V,Z),B(V)
55 DIM G(V),D(X),TR(Z)
60 REM I-ROW INDEX. J-COLUMN INDEX
70 FOR I=1TOY
80 PRINT"FLASK";I
81 PRINT#-2, "FLASK";I;
90 FOR J=0TOZ
100 INPUT"CONT FL VAL":C(I,J)
105 PRINT#-2,C(I,J);
110 NEXT J
115 PRINT#-2, " "
120 NEXT I
124 PRINT#-2, " "
125 PRINT#-2, "RESPIR."
130 FOR I=1TOX
140 PRINT"FLASK":I
141 PRINT#-2,"FLASK";I:
150 FOR J=0TOZ
160 INPUT"RESP FL VAL";R(I,J)
165 PRINT#-2,R(I,J);
170 NEXT J
175 PRINT#-2, " "
180 NEXT :
184 PRINT#-2, " "
185 PRINT#-2, "EXPER."
190 FOR I=1TOV
200 PRINT"FLASK":I
201 PRINT#-2."FLASK":I;
210 FOR J=0TOZ
```

```
220 INPUT "EXPER FL VAL":E(I,J)
225 PRINT#-2,E(I,J):
230 NEXT J
235 PRINT#-2, " "
240 NEXT I
246 PRINT#-2, " "
247 PRINT#-2, " "
250 BM=0
260 FOR N=1TOX
270 INPUT "RESP FL BIOM VAL":BM(N)
280 BM=BM+BM(N)
290 NEXT N
300 BM=BM/X
310 FOR I=1TOV
320 INPUT "EXPER FL BIOM":B(I)
330 NEXT I
340 FOR J=1TOZ
350 C=0
360 FOR I=1TO Y
370 C=C+C(I,J)-C(I,J-1)
380 NEXT I
390 C(0,J)=(C/Y)*CF
400 NEXT J
410 FOR J=1TOZ
420 R=0
430 FOR I=1TOX
440 R+R+R(I,J)-R(I,J-1)
450 NEXT I
460 R(0,J)=(R/X)*CF
470 NEXT J
480 FOR J=1TOZ
490 TR(J)=(R(0,J)-C(0,J))/BM
500 NEXT J
510 FOR I=1TOV
520 FOR J=1TOZ
530 E(I,J-1)=(E(I,J-1)-E(I,J))*CF
540 NEXT J
550 NEXT I
560 FOR I=1TOV
570 FOR J=Z-1 TO 0 STEP -1
580 E(I,J+1)=E(I,J)
590 NEXT J
600 NEXT I
610 FOR I=1TOV
620 FOR J=1TOX
630 E(I,J)=((E(I,J)+C(0,J))/B(I))+TR(J)
640 NEXT J
650 NEXT I
660 FOR I=1TOV
670 E(I,0)=0
680 NEXT I
690 FOR J=1TOZ
700 E(0,J)=0
```

```
710 NEXT J
720 FOR I=1TOV
730 FOR J=1TOZ
740 E(I,0)=E(I,0)+E(I,J)
750 NEXT J
760 E(I,0)=E(I,0)/Z
770 NEXT I
780 FOR J=1TOZ
790 FOR I=1TOV
800 E(0,J)=E(0,J)+E(I,J)
810 NEXT I
820 E(0,J)=E(0,J)/V
830 NEXT J
840 MC=0:MR=0
850 FOR J=1TOZ
860 MC=MC+E(0,J)
870 NEXT J
880 MC=MC/Z
890 FOR I=1TOV
900 MR=MR+E(I,0)
910 NEXT I
920 MR=MR/V
930 FOR I=1TOV
940 G(I)=0
950 FOR J=1TOZ
960 G(I)=G(I)+(E(I,J)-E(I,0)) 2
970 NEXT J
980 G(I)=SQR(G(I)/(Z-1)
990 NEXT I
991 PRINT#-2, "GROSS PHOTOSYNTHESIS"
992 PRINT#-2, " "
994 PRINT#-2, " "
995 FOR I=1TOV
996 PRINT#-2,"FLASK";I:
997 FOR J=0TOZ
998 E(I,J)=INT(10 1*E(I,J)+.5)/10 1
999 PRINT#-2, E(I,J):
1000 NEXT J
1001 PRINT#-2, " ";E(I,0),G(I),B(I)
1002 NEXT I
1003 PRINT#-2, " "
1004 PRINT#-2, " "
1009 DG=0
1010 FOR I=1TOV
1020 DG=DG+(E(I,0)-MR 2
1030 NEXT I
1040 MD=SQR(DG/(V-1))
1045 REM AVERAGE TRUE RESP
1050 RP=0
1060 FOR J=1TOZ
1070 RP=RP+TR(J)
1080 NEXT J
1090 RP=RP/Z
```

```
1093 REM S.D. FOR TRUE RESP
1095 RS=0
1100 FOR J=1TOZ
1110 RS=RS+(TR(J)-RP) 2
1120 NEXT J
1130 SR=SQR(RS/(Z-1))
1140 FOR I=1TOX
1150 FOR J=1TOZ
1160 R(I,J-1)=(R(I,J)-R(I,J-1))*CF
1170 NEXT J
1180 NEXT I
1190 FOR I=1TO X
1200 FOR J=Z-1 TO 0 STEP -1
1210 R(I,J+1)=R(I,J)
1220 NEXT J
1230 NEXT I
1235 REM CALC CORR RESP/MG
1240 N=0
1250 FOR I=1TOX
1260 N=N+1
1270 FOR J=1TOZ
1280 R(I,J)=(R(I,J)-C(0,J))/BM(N)
1290 NEXT J
1300 NEXT I
1310 FOR I=1TOX
1320 R(I,0)=0
1330 NEXT I
1335 REM AVE EACH RESP FLASK
1340 FOR I=1TOX
1350 FOR J=1TOZ
1360 R(I,0)=R(I,0)+R(I,J)
1370 NEXT J
1380 R(I,0)=R(I,0)/Z
1390 NEXT I
1395 REM S.D. EACH RESP FLASK
1400 FOR I=1 TO X
1410 D(I)=0
1420 FOR J=1TOZ
1430 D(I)=D(I)+(R(I,J)-R(I,0)) 2
1440 NEXT J
1450 D(I)=SQR(D(I)/(Z-1))
1460 NEXT I
1465 REM CALC MEAN OF MEANS & S.D. FOR RESP
1470 RM=0
1480 FOR I=1TOX
1490 RM=RM+R(I,0)
1500 NEXT I
1510 RM=RM/X
1520 RD=0
1530 FOR I=1TOX
1540 RD=RD+(R(I,0)-RM) 2
1550 NEXT I
1551 RS=SQR(RD/(X-1))
```

```
1552 PRINT#-2, " "
1553 PRINT#-2, " "
1554 PRINT#-2, "COLUMN AVERAGE"
1555 PRINT#-2, " "
1556 FOR J=1TOZ
1557 E(0,J)=INT(10 1*E(0,J)+.5)/10 1
1558 PRINT#-2, E(0,J):
1559 NEXT J
1560 PRINT#-2, " "
1561 PRINT#-2, "RESPIRATION"
1562 PRINT#-2, " "
1563 N=0
1564 FOR I=1TOX
1565 N=N+1
1566 PRINT#-2,"FLASK";I:
1567 FOR J=0TOZ
1568 R(I,J)=INT(10 1*R(I,J)+.5)/10 1
1569 PRINT#-2,R(I,J):
1570 NEXT J
1571 PRINT#-2, R(I,0);D(I);BM(N)
1572 NEXT I
1573 PRINT#-2, " "
1575 RPINT#-2, "TRUE RESPIRATION AVERAGE AT EACH READING"
1576 PRINT#-2, " "
1577 FOR J=1TOZ
1578 TR(J)=INT(10 1*TR(J)+.5)/10 1
1579 PRINT#-2, TR(J):
1580 NEXT J
1581 RP-INT(10 1*RP+.5)/10 1
1582 PRINT#-2, RP:SR
1590 PRINT#-2, " "
1591 PRINT#-2, " "
1592 PRINT#-2, "CONTROL AVERAGE EACH READING"
1593 PRINT#-2, " "
1594 FOR J=1TOZ
1595 C(0,J)=INT(10 1*C(0,J)+.5)/10 1
1596 PRINT#-2, C(0,J):
1597 NEXT J
1598 PRINT#-2, " "
1599 PRINT#-2, " "
1600 REM VARIABLE LIST
1610 PRINT#-2,"CORRECTION FACTOR=";CF
1620 PRINT#-2, "# OF CONTROL FLASKS =";Y
1630 PRINT#-2, "# OF RESPIRATION FLASKS =";X
1640 PRINT#-2, "# OF EXPERIMENTAL FLASKS =";V
1650 PRINT#-2, "# OF READINGS =";Z
1655 PRINT#-2, " "
1656 PRINT#-2, " "
1660 PRINT#-2, "I=ROW INDEX, J=COLUMN INDEX"
1670 PRINT#-2, "C(I,J)= INDIVIDUAL CONTROL READINGS"
1680 PRINT#-2, "C(0,J)= AVERAGE CORRECTED CONTROL VALUE"
1690 PRINT#-2, "R(I,J)= INDIVIDUAL RESPIRATION READINGS"
1700 PRINT#-2, "R(0,J)= AVE. CORR. RESP. FOR EACH COLUMN READING"
```

```
1710 PRINT#-2, "R(I,J)= AVE. CORR. RESP. FOR EACH FLASK"
1720 PRINT#-2, "TR(J)= TRUE RESP. FOR EACH COLUMN READING"
1730 PRINT#-2, "BM(N)= INDIV. RESP. FLASK BIOMASS"
1735 PRINT#-2, " "
1736 PRINT#-2, " "
1740 PRINT#-2, "MEAN OF TRUE RESP.=RP=";RP
1750 PRINT#-2, "AVE. BIOMASS FOR RESP. FLASKS =BM=";BM
1760 PRINT#-2, "MEAN OF MEANS FOR RESP. FLASKS=RM=";RM
1770 PRINT#-2, "SR=STANDARD DEVIATION OF TRUE RESP.=";SR
1780 PRINT#-2, "RS=S.D. FOR RESP. MEAN OF MEANS =";RS
1785 PRINT#-2, " "
1786 PRINT#-2, " "
1790 PRINT#-2, "E(I,J)=INDIV. EXP. READINGS;BECOMES GROSS PS VALUE"
1800 PRINT#-2, "E(I,0)= AVE. GROSS PS FOR EACH FLASK"
1810 PRINT#-2, "E(0,J)= AVE. GROSS PS FOR EACH READING"
1820 PRINT#-2, "B(I)= BIOMASS VALUES FOR EXP. FLASKS"
1821 PRINT#-2, "G(I)=S.D. FOR GROSS PS READINGS OF EACH FLASK"
1822 PRINT#-2, "D(I)=S.D. FOR EACH RESP. FLASK"
1825 PRINT#-2, " "
1826 PRINT#-2, " "
1830 PRINT#-2, "MC=MEAN OF MEANS FOR GROSS PS COLUMN READINGS =";MC
1840 PRINT#-2, "MR=MEAN OF MEANS FOR GROSS PS FLASK AVE.=";MR
1860 PRINT#-2, "MD=S.D. FOR MEAN OF MEANS FOR GROSS PS=";MD
```

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