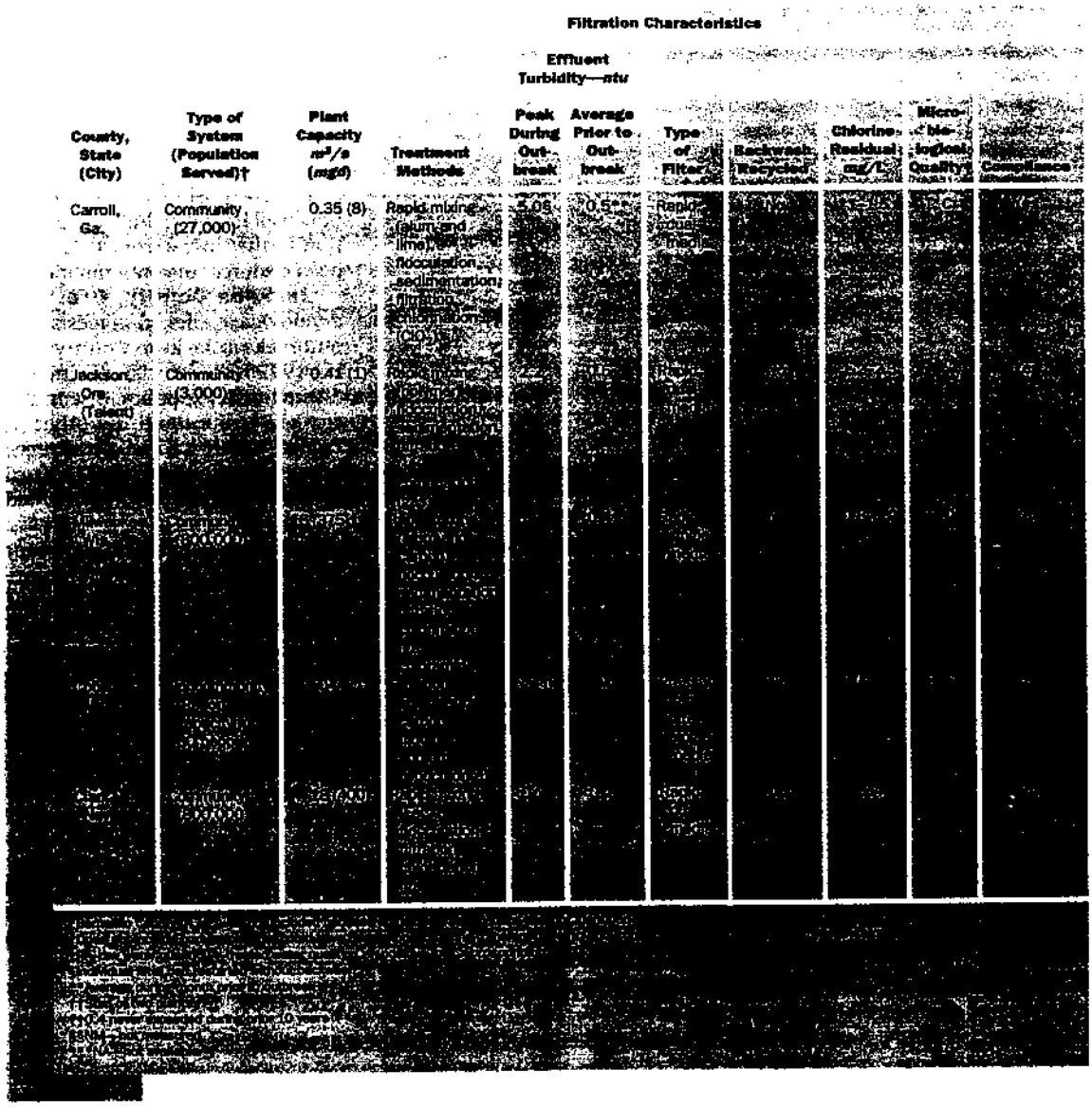
TABLE 3 Characteristics of surface water treatment systems*



County, State (City)	Type of System (Population Served)†	Plant Capacity m^3/s (*mgd*)	Treatment Methods	Filtration Characteristics: Effluent Turbidity—*ntu*: Peak During Outbreak	Filtration Characteristics: Effluent Turbidity—*ntu*: Average Prior to Outbreak	Filtration Characteristics: Type of Filter	Filtration Characteristics: Backwash Recycled	Filtration Characteristics: Chlorine Residual mg/L	Filtration Characteristics: Microbiological Quality‡	Filtration Characteristics: Filtration Compliance
Carroll, Ga.	Community (27,000)	0.35 (8)	Rapid mixing (alum and lime), flocculation, sedimentation, filtration, [illegible]	5.05	0.5**	Rapid [illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Jackson, Ore. (Talent)	Community (3,000)	0.41 (1)	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

The raw water source for the city of Talent is a river water. The river receives treated wastewater (activated sludge process and chlorinated) 8 km (5 mi) upstream and can be affected by runoff from adjacent agricultural land.[21] The outbreak coincided with drought conditions that limited the dilution of the wastewater by natural stream flow and with a small rainfall that could have contributed contaminated runoff.

The Talent water treatment system consisted of an infiltration-gallery intake buried under the stream bed, polymer coagulant addition, rapid mix unit, flocculation, tube settlers, dual-media filters, granular activated carbon filters, and postchlorination. Review of the plant during the outbreak revealed an inoperative rapid mix unit, inoperative filter-rate control valves, deficient filter operation, and poor effluent water quality (turbidity > 1 ntu). Water from the treatment plant was tested for *Cryptosporidium*, but the sample could not be fully examined because of interferences from excessive algal organisms. Improvements to the treatment plant included equipment repairs, use of alum as the primary coagulant, prechlorination to prevent the growth of algae in the filters, and improved filter operation. Correction of the deficiencies resulted in substantial improvement in treated water quality, and these improvements coincided with the end of the Jackson County outbreak period.[21]

TABLE 4 Corrective actions for systems treating groundwater and surface water*

County, State (City)	Corrective Action
Systems using groundwater	
Bexar, Texas	Old well decommissioned; water supplied is a chlorinated groundwater from the San Antonio treatment system.
Berks, Pa.	Old well decommissioned; water now supplied from a new well drilled in a location farther away from suspected sources of contamination
Jackson, Ore. (Medford)	None, except that the distribution system was flushed with a chlorinated and filtered river water shortly after the outbreak
Yakima, Wash.	Old well decommissioned; water supplied from a new well and treated using UV technology and a two-stage filter
Walla Walla, Wash.	Affected well decommissioned; water supplied entirely by another preexisting well
Systems using surface water	
Carroll, Ga.	Installation of baffled flocculators, increased monitoring of filter performance, polymer addition as a coagulant aid, improved chemical dosing, and improved operational practices
Jackson, Ore. (Talent)	Equipment repairs, use of alum as the primary coagulant, prechlorination to prevent the growth of algae in filters, and improved filter operation
Milwaukee, Wis.	Stricter operational practices including stricter monitoring of coagulant dosing and filter operation; renovation of filter units; filter backwash is sent to waste; proposed long-term improvements include extending raw water intake pipe and addition of ozone disinfection facilities
Cook, Minn.	Addition of a bag filter (5-μm effective pore size); raw water supply line replaced; a submersible pump added at the raw water inlet to assure a positive pressure on the supply line
Clark, Nev.	Increased monitoring frequency for *Cryptosporidium*; stricter effluent criteria; polymer addition to filter backwash

Milwaukee County, Wis. The largest waterborne outbreak in US history occurred during spring 1993 in Milwaukee.[6] Considerable epidemiologic and environmental evidence indicates that the outbreak was caused by a large amount of *Cryptosporidium* passing through one of the drinking water treatment facilities of the Milwaukee Water Works.[22] Water frozen for commercial ice production during the outbreak tested positive for *Cryptosporidium*.

At the time of the outbreak, Milwaukee Water Works served a population of 840,000. Within the greater Milwaukee area (population 1.6 million), approximately 403,000 people became ill,[22] and 4,400 people were hospitalized.[6] A mortality study performed by the Wisconsin Division of Health found that the outbreak was responsible for the premature deaths of at least 69 individuals, most of whom were HIV-positive.[23]

During the outbreak, Milwaukee Water Works had two treatment plants in operation. The plant located in the northern part of the city had a capacity of 12 m^3/s (275 mgd); the plant located in the southern area had a capacity of 4.4 m^3/s (100 mgd). These plants supply water at opposite ends of a common distribution system. The southern plant was implicated as the source of the outbreak because of unprecedented increases in effluent turbidity (maximum level = 1.7 ntu) and the high attack rates (>50 percent) within areas it served.

Both plants draw water from Lake Michigan and utilize alum coagulation, flocculation, sedimentation, rapid sand filtration, and chlorination treatment processes. Accessory treatment processes at the southern plant included chlorine and permanganate addition at the raw water intake (15 m [50 ft] deep) for taste and odor control and to prevent pipe fouling, carbon addition in the rapid mix unit for taste and odor control, fluoride addition, prechlorination using chlorine gas, and residual chlorination via chloramines. Why the southern plant failed to maintain low turbidity levels is unclear; improper coagulant dosage and the recycling of filter backwash water, however, are suspected to have contributed to the increase in turbidity.[22]

...case emphasizes the importance of raw ...er protection and maintenance of optimal water treatment at all times.

The precise source of contamination in the Milwaukee outbreak was not identified; possible sources of *Cryptosporidium* included cattle wastes, slaughterhouse wastes, and human sewage. It is speculated that rivers transported the oocysts from these sources to Lake Michigan during a period of high flow that resulted from the combined effects of spring rains and snowmelt runoff. Once within Lake Michigan, the

oocysts could then enter the intakes of Milwaukee Water Works treatment plants.[22]

At the time of the outbreak, Milwaukee Water Works was complying with federal and local standards. Since the outbreak, Milwaukee Water Works has developed much stricter standards and operational practices, including stricter monitoring of coagulant dosing and filter operation. Proposed long-term improvements to the water treatment processes include filter renovation, extension of the raw water intake away from suspected sources of contamination, and addition of ozone disinfection facilities.[24]

Yakima County, Wash. In April 1993, 10 people were exposed to untreated well water in Yakima County.[25] Seven contracted diarrheal symptoms, and three were confirmed cases of cryptosporidiosis. *Cryptosporidium* oocysts and other indicators of surface water contamination were found in the well water. Well water could have been affected by melting snow and spring rains contaminated by cattle, sheep, or elk feces.[25,26] Since the outbreak, the old well has been decommissioned, and water is pumped from a new well. Disinfection is now effected using ultraviolet technology and a two-stage filter (20 and 5 µm).[26]

Despite operational deficiencies, the treatment plant was complying with federal regulations at the time of the outbreak.

Cook County, Minn. In August 1993, 27 of 58 guests at a lake resort in Cook County became ill. An epidemiologic analysis found that consumption of drinking water was associated with the illness. The majority of stool samples tested were positive for *Cryptosporidium* and negative for other pathogens.

Drinking water was drawn from a lake at a depth of 8 m (25 ft). The lake water is pressure-filtered, chlorinated, and temporarily stored prior to distribution. Raw lake water tested positive for *Cryptosporidium*, but no oocysts were found in the treated water. The outbreak coincided with an episode of low water pressure within the distribution system. Backflow from a toilet facility and effluent from the resort's septic tank system were suspected sources of contamination.[27] Another possibility for contamination included leakage of septic tank effluent into a raw water intake line that flowed under negative pressure. Since the outbreak, a bag filter (5-µm effective pore size) has been added to the treatment scheme, the raw water intake line has been replaced, and a submersible pump has been added at the raw water inlet to assure a positive pressure on the supply line.[28]

Clark County, Nev. Epidemiologic evidence indicates that drinking tap water was the major risk factor for cryptosporidiosis during the Las Vegas outbreak in 1994,[29,30] and the outbreak did not coincide with changes in water plant operations or malfunctions within the distribution system. During the outbreak, all water samples taken at the plant and within the distribution system were negative for *Cryptosporidium*. Expert reviews of the epidemiologic study support that tap water was a major risk factor; nevertheless, the study has been criticized.[31]

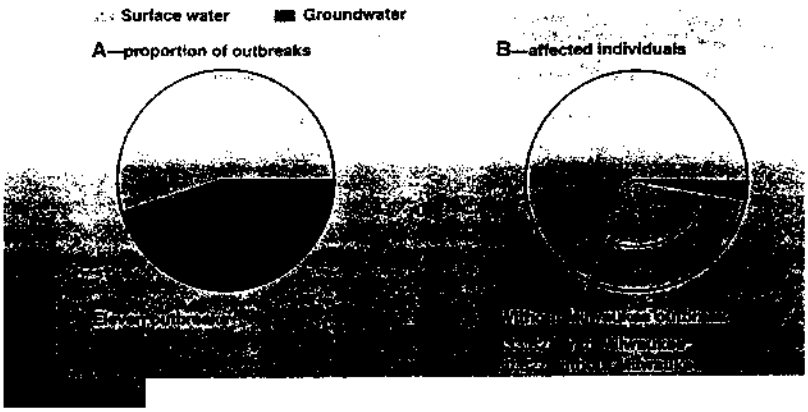
The total number of laboratory-confirmed cases associated with the outbreak was 103. Seventy-eight of these occurred during the epidemiologic study period. Of the 78 cases, 63 were HIV- or AIDS-infected patients (61 adults and 2 children), 11 were non-HIV- or AIDS-infected children, and the remaining 4 were non-HIV- or AIDS-infected adults. Two of the non-HIV adults were undergoing immunosuppressive treatments at the time of the outbreak.[29]

Of the 78 cases, 41 had died by May 1995, and at least 20 (all HIV-infected) had cryptosporidiosis listed as the cause of death on their death certificates. The degree of immunosuppression, or CD4 cell count, was an important factor in the development of disease in the HIV-infected population. Although the majority of laboratory-confirmed cases occurred among the HIV-infected population, evidence suggests that the outbreak may have extended into the general community.[6,30]

Influent water is drawn from Lake Mead at a depth of 40 m (131 ft). Lake Mead is a major reservoir on the Colorado River and was formed when Hoover Dam was constructed. The quality of the influent water into the treatment plant is considered pristine. The average turbidity of the raw water from January 1993 to June 1995 was 0.14 ntu with a high of 0.3 ntu,[32] and the plant consistently produces effluent turbidities of < 0.1 ntu.[31] The plant serves a population of 800,000 and treats 17.5 m^3/s (400 mgd) of water by direct filtration with ferric chloride as the primary coagulant, followed by radial-flow flocculators and dual-media filters. Disinfection is effected through pre- and postchlorination.[32,33] The water treatment facility is a state-of-the-art facility that is fully automated and computerized.[29]

Possible sources of contamination included a treated wastewater discharge to the lake located 10 km (6 mi) from the raw water intake, sewage from boats moored at a nearby marina, and a nearby bathing beach.[29] Furthermore, the outbreak roughly coincided with the annual turnover of the lake caused by the weakening of the lake's thermocline.[31] Postcontamination of treated water was considered; storage reservoirs are covered and secured, however, and no major system disruptions occurred during the outbreak period.[31] Possible treatment deficiencies

FIGURE 1 Comparison of surface water versus groundwater for proportion of outbreaks and affected individuals



included the passage of oocysts through filter units, especially during the ripening stage, and the recycling of backwash water to the head of the plant.

Since the outbreaks, the following procedural changes have been implemented: a total count of 10 particles/mL (2.5–150-μm size range) is the acceptable limit, a polymer is added to filter backwash to enhance its treatment prior to recycle, and the frequency of monitoring for *Cryptosporidium* is increased. From July 1994 to February 1996, the average number of presumptive[34] *Cryptosporidium* oocysts detected in the raw, backwash, and finished water were 11/100 L, 59/100 L, and < 1/100 L, respectively. As of February 1996, one confirmed[34] *Cryptosporidium* oocyst was detected in the backwash water.[32]

Walla Walla County, Wash. In 1994, a cryptosporidiosis outbreak in Walla Walla County resulted in 86 confirmed cases. Consistent with a waterborne outbreak, the confirmed cases corresponded to people of all ages living throughout a particular distribution system (Hydro 9 District) serving a population of 227. Results of an epidemiologic and environmental investigation indicated that contaminated well water was the source of the outbreak.[25] Epidemiologic evidence also pointed to a greater risk of contracting the illness with consumption of greater quantities of unboiled well water.

The water supply consisted of two artesian wells; well 1 was 150 m (500 ft) deep, and well 2 was 180 m (600 ft) deep. The water supply was generally untreated; immediately prior to the outbreak, however, the water was chlorinated because fecal coliform had been detected. The wells are near cattle-grazing areas and were adjacent to an irrigation system that distributes treated wastewater. The wastewater undergoes secondary treatment, sand filtration, and chlorination.[35] Upon inspection, treated wastewater from the damaged irrigation system was observed to seep into well 1. *Cryptosporidium* was found in well 1 water and in the treated wastewater.

After the outbreak, the irrigation system was repaired, and well 1 was decommissioned. The well water for the community now comes entirely from well 2.[25]

Alachua County, Fla. In July 1995, at a camp in Alachua County, Fla., 72 people out of a group of 104 became ill. All symptomatic individuals tested positive for *Cryptosporidium*.[36] The source of contamination was presumed to have been inadequate backflow prevention at the point of distribution. It is suspected that contaminated water from a garbage-can washer backflowed into the camp's kitchen plumbing system. A hose attached to the water supply on the garbage-can washer could have come in contact with wastewater and under negative pressure could have drawn wastewater into the distribution system. Wastewater within the garbage-can washer tested positive for *Cryptosporidium* oocysts. Since the outbreak, a backflow prevention device has been added to the system, and access to the garbage-can washer area has been restricted.

Discussion

Outbreaks of cryptosporidiosis have occurred in both small and large communities. Outbreaks were noted in small rural communities served by small water supply systems (e.g., Walla Walla and Yakima counties) and in heavily urbanized cities with water supply systems serving populations of several hundred thousand (e.g., Milwaukee and Clark counties). Raw water sources included both surface water (rivers and lakes) and groundwater (springs and pumped sources). Of the total number of outbreaks, roughly half occurred with groundwater sources. However, the majority of individuals affected (98 percent in all outbreaks, 67 percent without the Milwaukee outbreak)* were

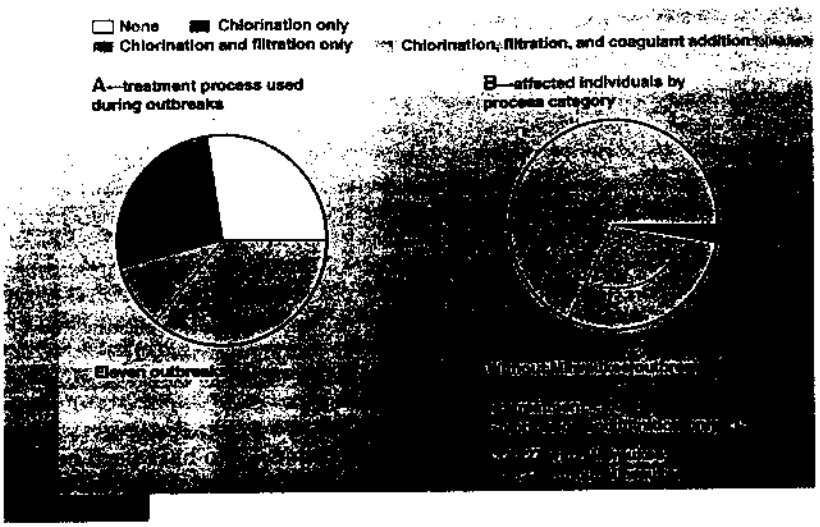
Most of the people affected by outbreaks of waterborne disease were served by surface water supplies.

*Because the Milwaukee outbreak overwhelms individual count values, computations are done both with and without its inclusion. Even with the exclusion of the Milwaukee data, however, the majority of affected individuals were served by surface water supplies.

served by surface water supplies (Figure 1).

Wastewater was implicated as a source of raw water contamination in four of the 11 outbreaks (Bexar, Carroll, Jackson [Talent], and Walla Walla counties). In two outbreaks, inadequate backflow prevention was suspected as a cause of contamination (Cook and Alachua counties). Thus, situations leading to roughly half of the outbreaks emphasize the importance of protecting drinking water from wastewater contamination at the raw water source and within the distribution system.

FIGURE 2 Water treatment processes used during outbreaks and number of affected individuals by treatment process category



For outbreaks in which no strong evidence of a wastewater connection existed, environmental factors were implicated as potential contributors to contamination. For Milwaukee, Clark, Carroll, and Bernalillo counties, which were all associated with surface water sources, nearby agricultural land uses and livestock grazing areas were implicated as possible sources of *Cryptosporidium*, and transport of oocysts from these areas could have been facilitated by snowmelt, runoff from rainfall, and lake turnover. For Berks, Jackson (Medford), and Yakima counties, all of which drew from groundwater sources, evidence implied that the groundwater could have been affected by surface water infiltration. The effect of environmental factors and nonpoint pollution can be partly controlled through watershed management practices designed to protect raw water supplies. Contamination of groundwater sources by surface water further emphasizes the need to include infiltration areas within watershed management plans.

Groundwater supplies were either untreated or chlorinated. To date, no reported outbreaks have occurred for systems in which groundwater was treated by softening or filtration processes. Treatment of surface water ranged from no treatment (Bernalillo County) to full conventional coagulation, filtration, and chlorination processes (Carroll, Jackson [Talent], and Milwaukee counties). Roughly half of the outbreaks occurred for systems in which treatment was nonexistent or limited to chlorination only; however, the majority of affected individuals (98 percent in all outbreaks, 67 percent without the inclusion of the Milwaukee outbreak) were served by large treatment systems employing coagulant addition, chlorination, and filtration (Figure 2). The large fraction of estimated cases of illness in people served by plants utilizing chlorination, coagulant addition, and filtration is consistent with Figure 1 because the raw water sources for these plants is surface water.

Disinfection for all treated waters was achieved by chlorination (i.e., chlorine gas, liquid hypochlorite, chlorine dioxide, or chloramines). During all outbreaks, the chlorination systems appeared to have been operating satisfactorily and were providing a measurable residual. Apparently, conventional chlorination processes alone are incapable of providing an adequate level of public health protection against *Cryptosporidium*, especially when the raw water supply is of poor quality. These observations are consistent with recent investigations that have found that the chlorine concentrations (either free chlorine or chloramine) needed for a measurable amount of *Cryptosporidium* inactivation[37] are well above concentrations used during conventional treatment plant operations and would result in unacceptably high chlorine residuals.

Five of the outbreaks—Carroll, Jackson (Talent), Milwaukee, Cook, and Clark counties—were associated with filtered drinking waters. Because effluent turbidities are not available for the Cook plant, this discussion of filtered drinking water is limited to four treatment systems. Of the remaining four treatment systems, three—Carroll, Jackson (Talent), and Milwaukee—were experiencing operational deficiencies, high effluent turbidities, or both, at the time of the outbreaks. All three plants utilized a full coagulation process that includes, in addition to rapid mix and flocculation, a sedimentation basin prior to the filter unit. Whether correction of deficiencies could have avoided an outbreak is unknown, but it is certain that these outbreaks occurred when treatment was suboptimal. Until the effectiveness of treatment processes in removing *Cryptosporidium* has been proved, plants should be operated at optimum levels to maximize the protection afforded by each existing process. Nonoperational or malfunctioning units and sudden increases in turbidities (which can be associated with poor coagulation and filtration processes) should be cause for alarm.

The system associated with the remaining outbreak (Clark) employed a direct filtration scheme. Direct filtration is similar to a full coagulation process except that treated water is not subject to a sedimentation process prior to filtration. Backwash water at the Clark County plant, however, is settled before being returned to the head of the plant.

The Clark County outbreak was the only outbreak associated with a filtered drinking water for which no treatment deficiencies were noted. The epidemiologic investigation conducted in response to this outbreak documented an association between the development of disease and the degree of immunosuppression among the HIV population. Therefore, this outbreak suggests that even at optimum treatment levels, certain subpopulations, especially the severely immunocompromised, may be at risk for contracting cryptosporidiosis from a public water supply.

In response to these outbreaks, regulations and current treatment processes are being reevaluated to assure that public health is protected. The Centers for Disease Control and Prevention, in conjunction with the US Environmental Protection Agency (USEPA), has issued a press advisory recommending that immunocompromised populations take extra precautions to reduce the risk of infection from *Cryptosporidium* in drinking water.[38] In May 1996, USEPA promulgated the Information Collection Rule,[34] which is intended to collect needed information for proposing and enforcing future regulations that will target treatment for *Cryptosporidium*. The Enhanced Surface Water Treatment Rule,[39] scheduled to be promulgated in 2000, proposes reduction criteria based on the numbers of *Cryptosporidium* oocysts in raw water supplies. The promulgated requirements would apply to all public water systems that use surface water or groundwater under the direct influence of a surface water and that serve 10,000 people or more. A provision for sanitary surveys is included for public systems that serve fewer than 10,000 people.

In addition, research is ongoing to determine inactivation and removal of *Cryptosporidium* by various treatment methods. Ozone[37] represents a promising alternative for disinfection. Dissolved-air flotation,[40] membrane filtration, and ultrafiltration[41] have been shown to physically remove considerable numbers of oocysts from water. Direct filtration has also been shown to effect significant removals after a filter-ripening period.[42]

Summary and conclusion

Recent outbreaks of cryptosporidiosis emphasize the importance of protecting drinking water from the effects of wastewater and nonpoint sources of contamination. For raw waters of poor quality, chlorination processes alone appear incapable of providing an adequate level of public health protection against *Cryptosporidium*. It is not clear how much protection is provided by conventional sand filtration processes. Until an adequate level of performance is identified, filtration systems should be operated at optimum levels exceeding existing regulatory requirements. Existing regulations and water supply systems should be reevaluated for protection against cryptosporidiosis. Further research is needed on proper watershed management practices and on effective treatment methods and operational practices.

Situations leading to roughly half of the outbreaks emphasize the importance of protecting drinking water from wastewater contamination at the raw water source and within the distribution system.

Acknowledgment

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ATTACHMENT #6

PROTECTING PUBLIC HEALTH

Waterborne disease surveillance

Because current reporting systems have drawbacks, drinking water professionals should collaborate with the public health community to enhance approaches to waterborne disease surveillance.

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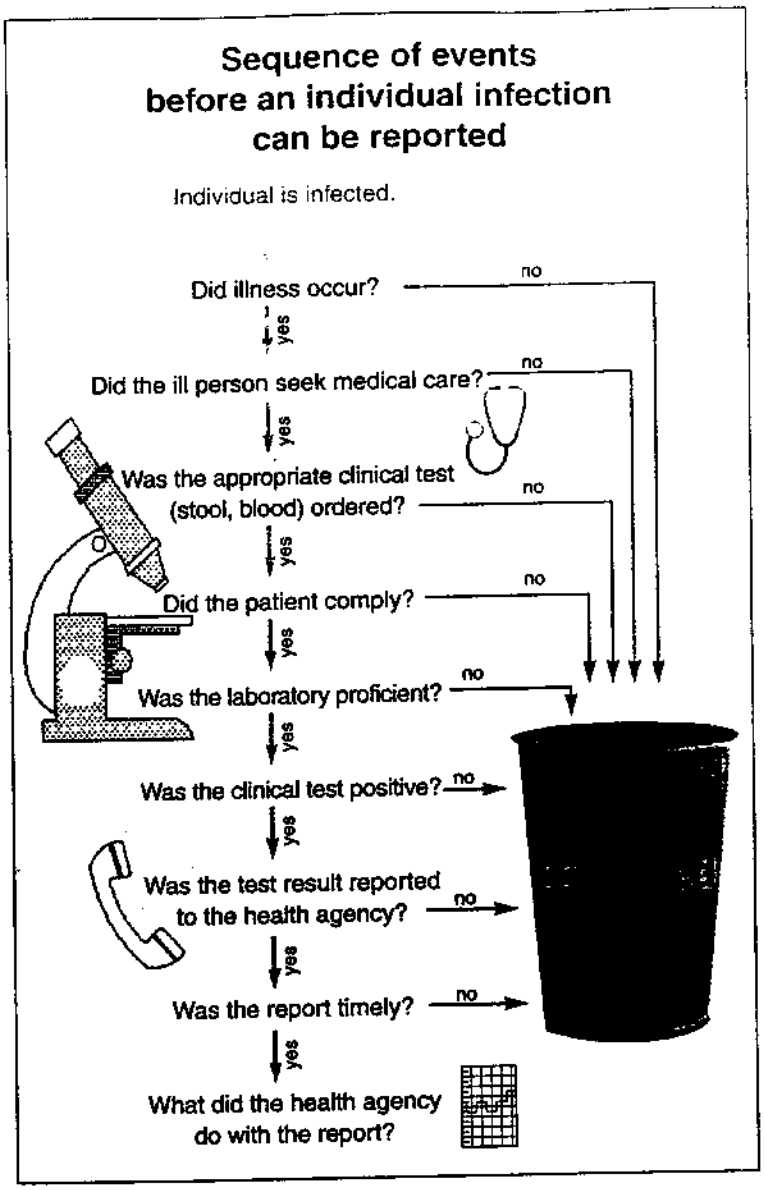
[illegible] disease surveillance [illegible] health departments. In addition, the authors recommend additional ways of improving disease surveillance and investigating endemic waterborne disease. Health care reform may reduce the effectiveness of the current system of laboratory-based disease reporting, but opportunities for enhanced disease surveillance may also emerge from these reforms. In addition, new laboratory technologies may make routine surveillance of enteric pathogen infections, rather than disease from these pathogens, increasingly feasible. The interests and information needs of the drinking water industry and its customers can best be assured by its informed participation in the debate over the design and goals of new disease surveillance programs.

Public health surveillance has played a key role in controlling the spread of communicable disease and identifying the need for modern public health practices such as the filtration and chlorination of drinking water supplies. Recent outbreaks of waterborne disease, however, have raised questions about the efficacy of current water treatment practices in preventing transmission of some enteric pathogens.[1–4] Outbreaks have been reported in areas that receive filtered, disinfected water that meets drinking water standards,[5–7] and an epidemiological study found that consuming drinking water derived from surface water sources, even if it meets water quality stan-



dards, may significantly increase the risk of enteric illness in the absence of an outbreak.[8] Of all enteric disease in the United States, a significant fraction may be caused by drinking water.[9]

As the need increases for better information about waterborne disease, it has been suggested that the US disease surveillance system is in a state of crisis and may possibly collapse.[10] State health departments often cannot dedicate any staff to enteric or waterborne disease surveillance.[11] This article discusses the key issues relating to disease surveillance and outbreak investigations. With limited public health resources available, it is important to carefully consider the goals of waterborne disease surveillance and to ensure that the information needs of the public, the regulatory agencies, and the industry are best served. Drinking water professionals need to understand the strengths and weaknesses of current surveillance and outbreak detection programs, because they may be required to participate in (and, perhaps, contribute funding to) these programs.

Current reporting systems could use improvement

Additional information is needed about the occurrence and causes of waterborne disease, both epidemic and endemic. The Centers for Disease Control and Prevention (CDC) has funded pathogen surveillance projects in selected states partly to improve surveillance for several potentially important waterborne agents.[12] In New York City, the Department of Environmental Protection (DEP), which is responsible for drinking water treatment and delivery, recently convened a panel of public health experts to evaluate current health department disease surveillance programs.[13] This panel recommended specific waterborne disease surveillance activities and epidemiological studies to determine endemic waterborne disease risks that may be associated with use of unfiltered surface water sources (see the sidebar on page 68). Efforts to improve New York's waterborne disease surveillance system are being funded by the DEP—the first time, to the authors' knowledge, that this has occurred for a drinking water utility.

Most current state and local health department surveillance programs are based on disease reporting by health care providers and clinical laboratories. However, several limitations of the system have been identified. The symptoms of most enteric waterborne diseases are similar (diarrhea and vomiting), and cases are only reported if the individual consults a physician and an etiologic agent is identified. Reporting is not required for all enteric diseases. Even when it is required, it is often incomplete. Newly recognized pathogens such as *Cryptosporidium* are often difficult to detect and diagnose. With current surveillance programs, even an outbreak resulting in many medically attended illnesses in a large city could be unrecognized. For example, if many health care providers over a large geographic area treated the patients, it is possible that no one provider would

Panel Recommendations for Waterborne Disease Surveillance

- Designate an individual who is specifically responsible for coordinating waterborne disease surveillance.
- Monitor visits to hospital emergency rooms for enteric disease.
- Monitor sales of prescription and nonprescription medications for diarrheal illness.
- Conduct special enteric disease surveillance studies of nursing home and retirement home populations.
- Conduct surveillance of managed health care populations.
- Conduct surveillance of high-risk populations.

recognize an increased occurrence of illness. In addition, the existence of an outbreak in a small but extremely susceptible subpopulation (e.g., patients with achlorhydria or reduced levels of gastric acid as a result of stomach surgery) probably would not be detected because of the small number of people at risk.

In the future, other factors may significantly reduce the effectiveness of traditional disease surveillance programs. Changes in health care access and delivery may reduce the number of patients seeking care and the chances that medically attended diseases are confirmed by laboratory tests. However, other changes in health care may present new opportunities for disease surveillance. Computerization of patient records, health care and laboratory workloads, prescription and nonprescription pharmaceutical sales, and calls to nurse hotlines are new tools that may bring about more effective and less costly disease surveillance. Technological advancements in the detection of antigen or antibodies specific to a pathogen in sera, stools, and other clinical specimens (e.g., saliva) may improve detection and identification of etiological agents. These diagnostic methods also allow the detection of infection in the absence of symptomatic disease.

What are the limitations of current disease surveillance systems?

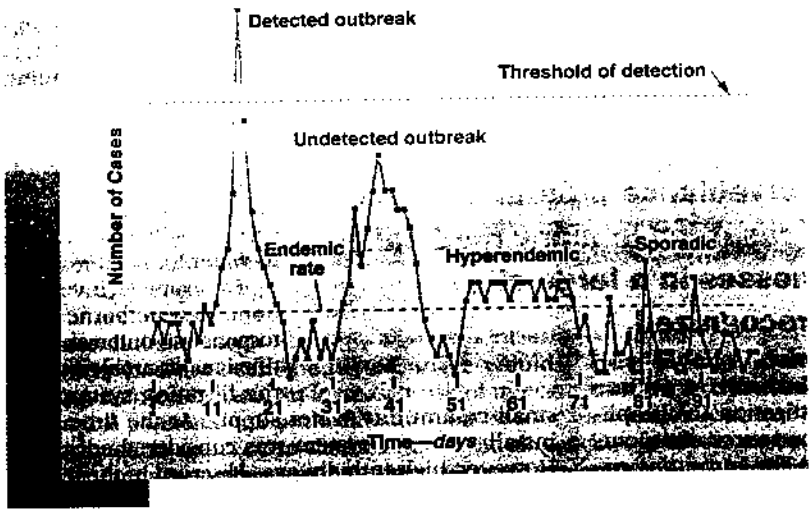
Detection of waterborne disease outbreaks depends partly on a state–federal system of notifiable or reportable diseases. State or local laws require the reporting of certain diseases, and primary responsibility for disease surveillance rests with state or local public health authorities. Most state surveillance systems are passive, in that disease reporting is primarily the responsibility of health care providers and diagnostic laboratories. Health care providers and laboratories usually receive little encouragement from the health department to report illnesses, and enforcement of reporting requirements is minimal. In an active surveillance system, some or all health care providers or laboratories are contacted routinely and asked for information about disease reports.[14] More cases are identified by active surveillance (see the sidebar on page 70).

Disease reporting is incomplete in both passive and active disease surveillance programs. Underreporting varies for each disease, among states, and among areas or populations within a state.[15] For example, reporting is likely to be more complete for severe diseases such as hemolytic *E. coli* than for milder infections such as Norwalk virus gastroenteritis. Because many enteric infections have the same symptoms, diagnosis cannot be made on the basis of symptoms alone; laboratory analysis of clinical specimens is required. Clinical laboratories tend to be much better at reporting their findings than are physicians,[14] and there can be great variations in reporting depending on the dedication of clinical laboratories and diagnosing physicians and the patient population they serve.[15]

Of enteric disease in the United States, a significant fraction may be caused by drinking water.

In addition to incomplete reporting of diagnosed illnesses, only a portion of all infections are medically diagnosed. Only a fraction of infections will lead to illness. Many infected persons can be unaware of their infection (see illustration, page 67). Whether infection leads to symptomatic illness depends on factors including dose and virulence of the organism and medical condition, age, and immune and nutritional status of the host. In other instances, the host experiences none of the classic symptoms. For example, sometimes children who have *Giardia* infections

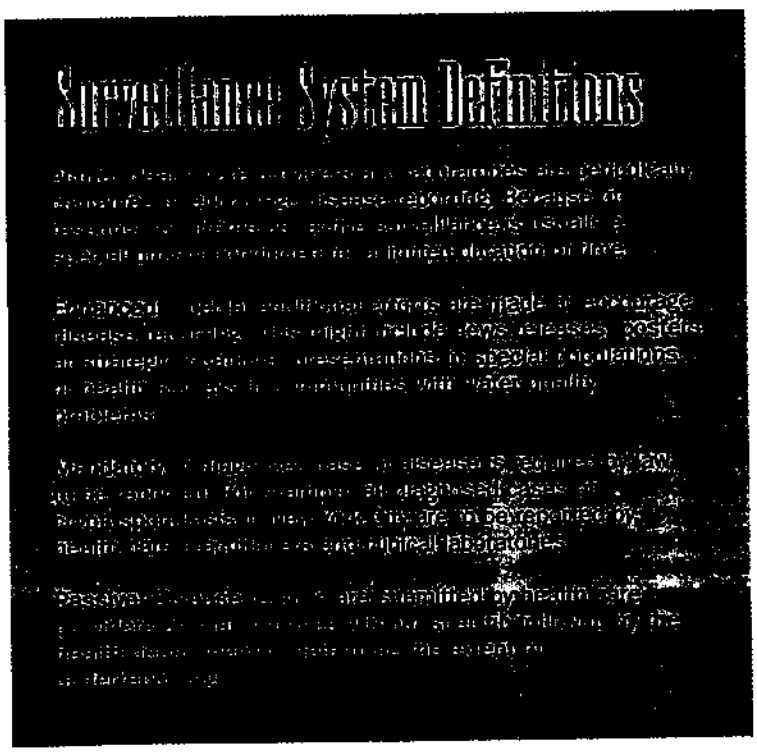
FIGURE 1 Endemic versus epidemic disease



fail to thrive but do not experience the classic symptoms of giardiasis. When symptoms occur, they may be mild or may resolve in a short time; the person may not seek medical care or may simply buy medicine at a pharmacy to alleviate the symptoms. For example, only half of all people infected with *Salmonella* report illness, and only 20 percent of those those who become ill consult a doctor.[16] If the person seeks health care, the physician may not correctly diagnose the infection, because symptoms are not often sufficiently specific to accurately identify the pathogen. If the infection is misdiagnosed and resolves itself, the patient may not seek additional health care, and no report of an infection is generated. Even when the diagnosis is correct and the appropriate medication is prescribed, a confirming laboratory test may not be ordered. If a laboratory test is ordered, however, many patients are unwilling to submit stool specimens for analysis. Because laboratories are the primary source of disease reports for surveillance systems, a report may never be filed if there is no laboratory-confirmed diagnosis. A stool or blood sample submitted for laboratory analysis can test negative because of analytical or specimen collection error or untimely collection or because the material submitted was, by chance, free of the pathogen.[17] Sometimes stool specimens are collected too late and the infectious agent is no longer being excreted; acute serum specimens may also be collected too late, and convalescent sera may not be collected for comparison. For those people infected with enteric parasites, single stools may often be free of the parasite or have insufficient numbers of parasites to assure laboratory detection. In some cases, even multiple stools may be pathogen-negative.

If enough reports of the same disease or pathogen come in to the health department at about the same time—and if the epidemiologist is alert to the increase—then an outbreak may be recognized. Depending on the incubation time of the illness and the time needed to perform diagnostic tests and report the results, outbreak investigations may begin weeks after the water is contaminated and the onset of the initial cases.

Many outbreaks are detected by an alert clinician. For example, in 1976, the son of a physician in Camas, Wash., returned from Russia with giardiasis. The physician later recognized that several of his patients had similar symptoms, which led to the identification of a waterborne giardiasis outbreak.[18] In Mil-



waukee, Wis., a pharmacist who noticed a dramatic increase in sales of antidiarrheal medication alerted the health department and television stations to the possible occurrence of an outbreak of enteric disease. Diarrheal illnesses reported to California and Arizona health agencies by 65 campers who had visited an Arizona park initiated an investigation that implicated contaminated water as the source of an outbreak affecting 1,850 people.[19] The fortuitous circumstances surrounding the detection of many outbreaks raise concerns about how many outbreaks are never detected. Small outbreaks may seldom be detected; even large ones may not be detected without active or enhanced disease surveillance programs.

With current surveillance programs, even an outbreak resulting in many medically attended illnesses in a large city could be unrecognized.

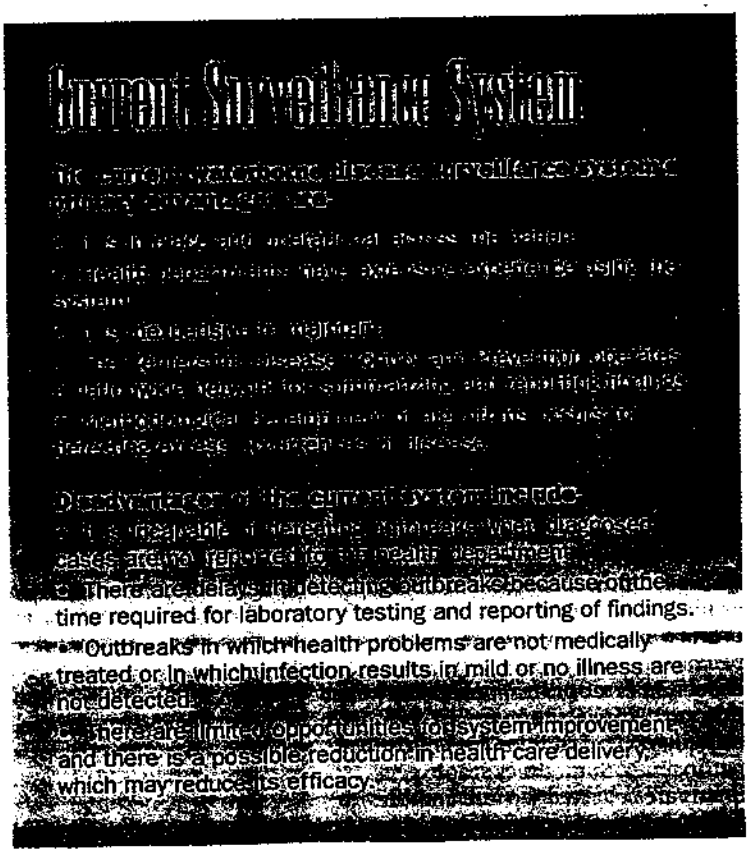
Studies to evaluate the efficacy of active surveillance programs provided mixed results. A three-state study of various approaches detected no additional waterborne disease outbreaks in Washington and Vermont;[20] however, in Colorado, a greater than threefold increase in the number of detected waterborne outbreaks occurred when surveillance was improved.[21]

Active and enhanced disease surveillance can only increase reporting of diagnosed illnesses from providers and laboratories. All of the other barriers to disease identification and reporting of cases to the public health agency will still remain (see illustration, page 67). If access to health care declines in the future or if physicians use fewer laboratory diagnostic services to reduce health care costs, the number of diagnosed reportable illnesses will decline despite the efforts of health departments to ensure that most diagnosed illnesses are reported.

How much surveillance is enough?

A waterborne disease outbreak is a newsworthy and politically important event. Many people may become ill, and affected populations may experience severe illness. As the outbreak is investigated and control measures are implemented, much is often learned about the cause of major failures in water treatment or distribution. However, when the water system's deficiencies have been corrected and the outbreak has been officially declared over, has the problem been solved? Or is disease continuing to occur at a reduced level not detectable by traditional surveillance activities? How much of this background level of disease can still be attributed to water?

For example, investigation of a waterborne cryptosporidiosis outbreak identified major problems with the filtration system of a small community water supply. At the time of its installation, the system was considered adequate. However, high turbidity was observed in the filtered water at the time of the outbreak. Optimization of the treatment process by consulting engineers allowed the plant to dramatically improve turbidity and pathogen removal, which reduced the number of new cases of cryptosporidiosis and officially ended the outbreak. Two years later, though, a serological survey of the town's residents suggested that *Cryp-*

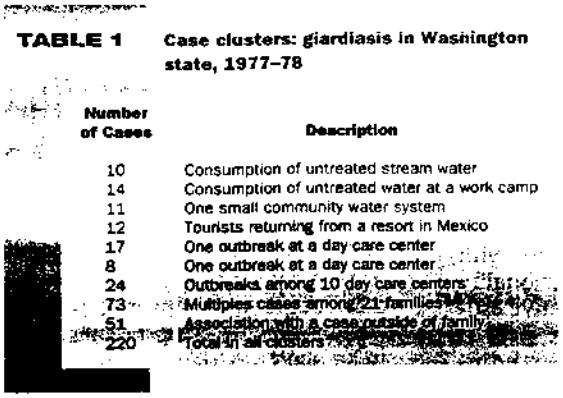


tosporidium infections were continuing. These infections were not accompanied by a disease outbreak, and because an epidemiological study was not conducted, it was not possible to determine whether these cases were associated with drinking water consumption.

This raises several philosophical and technical issues regarding waterborne disease surveillance. If the serological survey had not been conducted, there would have been no evidence of an increased risk of infection in this population. Should all outbreaks be followed by such a survey to determine continuing infection and its possible mode of transmission? Is evidence of continuing infection in the absence of symptomatic disease sufficient reason for further intervention? If water treatment has already been optimized, what intervention options remain? Can infection and disease be further reduced without new filtration or disinfection technology? Low levels of enteric disease over many years from exposure to waterborne microbes can place a much larger health burden on a community than disease associated with the detected outbreak, and this risk should be considered. Improving the sensitivity of a surveillance system to detect early signs of an outbreak is important, but detection of endemic waterborne risks before and after an outbreak is also important.

TABLE 1 **Case clusters: giardiasis in Washington state, 1977–78**

Number of Cases	Description
10	Consumption of untreated stream water
14	Consumption of untreated water at a work camp
11	One small community water system
12	Tourists returning from a resort in Mexico
17	One outbreak at a day care center
8	One outbreak at a day care center
24	Outbreaks among 10 day care centers
73	Multiples cases among 21 families
51	Association with a case outside of family
220	Total in all clusters



Clinical laboratories tend to be much better at reporting their findings than physicians.

Failure of a surveillance system to detect low levels of disease in a community may provide a false sense of security about waterborne transmission of disease. For example, why was an outbreak such as the one that occurred in Milwaukee, Wis., not preceded by many smaller outbreaks? Is it possible that in Milwaukee and other cities experiencing a large waterborne cryptosporidiosis outbreak, undetected smaller outbreaks had occurred previously? Or did a low prevalence of waterborne *Cryptosporidium* infection occur for years before each reported outbreak—and at the time of the detected outbreak, did more *Cryptosporidium* oocysts pass through the treatment system, or did a more virulent strain of the pathogen emerge? If these conditions occurred, relying on disease surveillance systems that can only detect large outbreaks will not provide early warnings to public health and water officials about waterborne disease risks and emerging new diseases. Use of disease surveillance systems that only detect large outbreaks is equivalent to basing the science of meteorology only on the study of hurricanes.

How much endemic disease can be attributed to drinking water?

Endemic disease is defined by CDC as a persistent low to moderate background level of disease occurrence. A high level of occurrence is called hyperendemic; an irregular pattern of occurrence is called sporadic (Figure 1). The threshold of detection for outbreaks is determined largely by the type of surveillance and resources devoted to it. Routine surveillance programs typically have poor sensitivity to detect small outbreaks (threshold of detection, in Figure 1). For most enteric infections, endemic disease rates during any year (Figure 1) consist of cases of disease from the statistical averaging of small, undetected outbreaks or clusters of infections. Endemic levels of disease probably do not remain constant over time or across geographic areas and may represent an important public health problem.

During this century, the importance of endemic infection and disease has become increasingly recognized. After World War I, an attempt was made to estimate the prevalence of parasitic infections in both the returning British soldiers and the British population remaining at home.[22] To the surprise of the researchers, a high prevalence of asymptomatic infection was found among people who had never left Britain. Later, a survey of Wise County, Va., in 1930 found that half of the population carried *Entamoeba histolytica* and that 38 percent carried *Giardia lamblia*.[23] More recently, a study to determine the incidence of *Cryptosporidium* infection among Peace Corps workers before they went overseas showed that almost 30 percent had increased antibody levels, suggesting they had experienced infection before leaving the United States.[24]

Using information from current disease surveillance systems, it is not possible to meaningfully compare endemic disease levels between areas or populations with different water systems. Such a comparison cannot address whether observed dif-

ferences in reported disease reflect differences in the completeness of reporting or differences in the occurrence of disease or infection. Nor can it address whether the observed differences are associated with water treatment or quality. Epidemiological studies must be specifically designed and conducted to address the association of endemic disease with water system type or water quality and must provide a quantitative estimate of disease risk.

Several epidemiologic studies have identified waterborne endemic disease risks in the absence of a reported waterborne outbreak. In New Zealand, the incidence of laboratory-confirmed giardiasis was higher in a population receiving chlorinated, unfiltered surface water than in a population receiving surface water treated by coagulation, flocculation, granular filtration, and chlorination.[25] In Vermont, a higher incidence of endemic giardiasis was found in municipalities using unfiltered surface water or wells than in municipalities with filtered surface water.[26] A recent study attempted to estimate how much endemic enteric illness was attributable to drinking water.[9] The fraction of illness attributable to drinking water was estimated by comparing self-reported "highly credible gastrointestinal illnesses" among people drinking treated municipal tap water and those drinking water from reverse osmosis filtration units. Although it cannot be completely ruled out that different rates of illness resulted from reporting biases, this study suggests that drinking treated municipal water may significantly contribute to endemic disease in at least one community.

Enhanced Surveillance System

The advantages of an enhanced waterborne disease surveillance system are:

- It may detect outbreaks if few patients seek health care or if the illness is so brief that health care is not needed.
- It detects outbreaks relatively quickly, because the time delay between the onset of symptoms and the purchase of over-the-counter drugs or calls to nurse hotlines is likely to be short.
- It is relatively inexpensive to maintain (especially if retail pharmacies are involved nationwide or standardized nurse hotline software is programmed for reporting).

Disadvantages of an enhanced system include:

- It will not usually identify the etiological agent because only symptoms are ascertained.
- Although it is inexpensive to maintain, it requires investment for initial computer programming and establishment of data-sharing agreements.
- False leads (possible outbreaks that result in cases investigation).

utbreak investigations may begin weeks after the water is contaminated and the onset of the initial cases.

Can outbreak investigations be used to estimate risks?

Epidemic disease is the increased occurrence or clustering of a specific illness, and data about epidemic waterborne disease are available from reported outbreaks. Between 1971 and 1994, 737 waterborne disease outbreaks were documented in the United States.[5–7,27] Almost half of these were caused by unknown etiological agents that cause acute gastrointestinal illness. In the remaining outbreaks, etiologic agents (viruses, bacteria, protozoa, or chemicals) were identified. Identification of an etiologic agent largely reflects the ability to diagnose these infections and the general interest in collecting and analyzing clinical specimens for a specific agent. However, outbreaks do provide some indication of the relative importance of different agents for consideration by water officials. Outbreaks also provide information about attack rates for people exposed to contaminated drinking water and the severity (e.g., death or hospitalization) of waterborne disease. However, it can be misleading to compare the frequency of waterborne outbreaks and number of associated cases of illness with other outbreaks (e.g., day care center outbreaks) to estimate the relative importance of the two modes of transmission.

Only if outbreaks account for the majority of illnesses is the outbreak of primary interest—and for most waterborne pathogens, outbreaks account for only a small fraction of all illnesses. For example, in 18 months during the late 1970s in Washington, 1,347 laboratory-confirmed cases of giardiasis were reported to the state health department.[28] Extensive followup (Table 1) showed that clusters or possi-

ble small outbreaks accounted for only 16 percent of all cases of giardiasis reported during this time. The remaining cases were endemic, suggesting that "endemic giardiasis" was much more important than "epidemic giardiasis" in Washington. The importance of waterborne transmission both in the clusters and endemic cases remains unknown, because epidemiological studies were not conducted to assess this.

Information from investigations of waterborne outbreaks cannot reliably be used to estimate the burden of both epidemic and endemic waterborne disease. It is not known with any certainty to what extent waterborne outbreaks are underreported, and, as was noted previously, specific epidemiological studies are required to estimate the endemic risk attributable to drinking water. Potential problems of extrapolating the characteristics of disease reported in waterborne outbreaks to all waterborne disease include the following:

- If there is variation in the virulence of a pathogen, detected outbreaks may be predominantly caused by the more virulent strains of the pathogen. This may overestimate severe morbidity or mortality associated with the pathogen.
- If only reported outbreaks are examined, the importance of drinking water as a route of transmission may be overestimated. Because of the large numbers of cases often involved, waterborne outbreaks may be more easily detected than other outbreaks. Even a severe outbreak in a day care center usually involves only a few cases. Clusters within a family usually involve only two or three cases.
- Outbreak detection is often more difficult for highly endemic diseases than for uncommon diseases. For example, two cases of cholera anywhere in the United States might be considered an outbreak, whereas 1,000 cases of cryptosporidiosis widely dispersed in a large US city during a week might easily be absorbed as background cases of diarrhea and not recognized as an outbreak.[13]
- Outbreaks of short-duration illnesses (e.g., those caused by some viruses) are more difficult to detect and study than are outbreaks of illnesses with long duration (e.g., giardiasis, shigellosis, hepatitis A). Therefore, when considering only information from outbreaks, the importance of acute, self-limited gastrointestinal illness of undetermined etiology and short duration may be underestimated relative to parasitic, some bacterial, or viral diseases with symptoms that last longer.

Should expanded surveillance programs monitor infection rather than illness?

That a carrier of infection can be asymptomatic has been known for some time (e.g., Typhoid Mary). However, the proportion of asymptomatic carriers for many infections has only recently been appreciated. The parasite prevalence surveys in Britain[22] and Virginia[23] found more asymptomatic infected individuals than expected. Even as late as 1952, in New Hope, Tenn., 10.6 percent of the general population was infected with *Giardia lamblia*.[24] Following a 1966 giardiasis outbreak in Aspen, Colo., a stool survey found that 5 percent of the population was infected with *Giardia*.[30] A survey in Boulder, Colo., also conducted following an outbreak, found the same prevalence of 5 percent.[31] Most of those who participated in these surveys were asymptomatic. A stool survey of one- to three-year-old children in Washington in 1980[32] found that 7 percent of the children were infected with *Giardia lamblia*. All participating children were reported as healthy at the time of the survey. During the 1960s and early 1970s, the monitoring of virus infections among a sample of people in selected US cities found that illness was reported in less than half of all enterovirus infections.[33]

When the water system's deficiencies have been corrected and the outbreak has been officially declared over, has the problem been solved?

New serological tools have been developed during the past 20 years to better monitor the prevalence of infections among the population. Even though infection may not result in illness, there are several reasons for considering the surveillance of infection rather than disease:

- Information about the incidence of infections can provide an early warning for outbreaks. Widespread, unrecognized transmission of infection may have devastating consequences for highly susceptible individuals in the population.
- Seroepidemiological studies of infection can better estimate the extent of endemic waterborne transmission for many etiologic agents, because these studies are statistically more powerful and can detect low risks. Results can expand understanding of the relative importance of the various modes of transmission if information is obtained about water exposures (drinking and recreational), other risk factors, and antibody status.
- Just as the total coliform test is useful to indicate potential water contamination, epidemiological studies of the waterborne transmission of infection—even when illness is predominantly asymptomatic—can provide critical information for evaluating water treatment systems and may help identify correctable problems in water source protection, treatment, or distribution.

Recommendations

Depending on the specific goals, several options are available for enhanced waterborne disease surveillance. The current national system of surveillance, based on diagnosed illness, has a long-established record for detecting some outbreaks (see the sidebar on page 70). Because this system is both inexpensive to maintain and currently operational, it has considerable support among public health practitioners. Monitoring for increased pharmaceutical sales of antidiarrheal medications, calls to nurse hotlines about diarrheal illness, or physician visits for gastrointestinal illness are potential alternatives to traditional disease surveillance programs (see the sidebar on page 72).

se of disease surveillance systems that only detect large outbreaks is equivalent to basing the science of meteorology only on the study of hurricanes.

If the goal of the surveillance system is to detect small outbreaks and provide early information about their occurrence, these alternative approaches have promise and should be evaluated for possible implementation as a nationwide system. No outbreak detection system, however, is likely to be of value for estimating endemic waterborne disease risks; specific epidemiological studies must be conducted.

It is becoming increasingly feasible to estimate the incidence or prevalence of antibody response to pathogens and to relate this information to various modes of transmission. Thirty years ago, the Virus Watch Program examined occurrences of viral infections among volunteers in selected communities.[33] Similar approaches using serodiagnostic tools to monitor the occurrence of *Giardia*[34] and *Cryptosporidium*[35] infections have recently been developed. As the usefulness of serodiagnostic and other tests is evaluated in the near future, cost-effective approaches to the widespread implementation of alternative surveillance activities will probably be available. Seroepidemiological studies may also provide the opportunity to learn why certain populations have higher endemic levels of disease than others and to greatly improve understanding of both drinking water and nondrinking water routes of transmission.

Changes in health care delivery may result in reduced use of diagnostic laboratory services. If so, this will also reduce the value of laboratory-based disease surveillance. Unfortunately, state and local health agencies that manage disease surveillance systems are often unaware of the opportunities to enhance surveillance. Projects to establish model disease surveillance and reporting systems could help state and local public health agencies develop relationships with large providers of health care.

To fully exploit these opportunities, a new public health partnership will be needed among health care providers, health maintenance organizations, and public health agencies. The drinking water industry should fully participate in this partnership. Drinking water professionals should actively reach out to the public health community to initiate, plan, and evaluate cost-effective approaches to waterborne disease surveillance. Low-cost infection and disease surveillance systems will provide an additional measure of safety to our drinking water systems, warning of possible problems. This surveillance, together with water quality monitoring and treatment plant optimization, can ensure that the risk of waterborne disease remains very low.

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ATTACHMENT #7

been shown to support HIV-1 replication (24), are among the first virus targets in primates after oral exposure and play a key role in subsequent virus spread.

In sum, cell-free SIV is significantly more transmissible through the oral route as compared with the intrarectal route. The use of omeprazole, which results in the neutralization of gastric acid, was not required for infection. Because formal oral titrations were not conducted to establish the minimal infective dose in neonatal macaques (10), we do not know whether the oral exposure route is significantly more permissive of SIV entry in neonates as compared with adults. Our data, together with the case reports of HIV-1 seroconversion after oral-genital sex only (5), have implications for HIV-1 transmission. We conclude that oral exposure to infectious virus in the absence of mucosal lesions carries the risk of infection and AIDS. Thus, unprotected receptive oral intercourse should be added to the list of risk behaviors for HIV-1 transmission.

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30. All animal experiments were approved by the Animal Care and Use Committees at the Tulane Regional Primate Research Center and at the Dana-Farber Cancer Institute, which take responsibility for humane care and use of laboratory animals. We are committed to comply with the Principles for Use of Animals, the *Guide for the Care and Use of Laboratory Animals*, the Provisions of the Animal Welfare Act, and other applicable laws and regulations. The center's statement of assurance is on file with the USPHS, Office for Protection from Research Risks. These facilities are accredited by the American Association for Accreditation of Laboratory Animal Care. Animals are anesthetized with ketamine before all procedures that require the removal of animals from their cages. No restraining devices are necessary during these procedures. When necessary, moribund animals are euthanized by iv inoculation of a lethal dose of sodium pentobarbital.

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Synergistic Activation of Estrogen Receptor with Combinations of Environmental Chemicals

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Certain chemicals in the environment are estrogenic. The low potencies of these compounds, when studied singly, suggest that they may have little effect on biological systems. The estrogenic potencies of combinations of such chemicals were screened in a simple yeast estrogen system (YES) containing human estrogen receptor (hER). Combinations of two weak environmental estrogens, such as dieldrin, endosulfan, or toxaphene, were 1000 times as potent in hER-mediated transactivation as any chemical alone. Hydroxylated polychlorinated biphenyls shown previously to synergistically alter sexual development in turtles also synergized in the YES. The synergistic interaction of chemical mixtures with the estrogen receptor may have profound environmental implications. These results may represent a previously uncharacterized level of regulation of estrogen-associated responses.

Reports of abnormal sexual development in reptiles (1, 2) or birds (3) as well as feminized responses in male fish (4–6) have suggested an association with environmental chemicals functioning as estrogens. Similar hypotheses have been advanced in relation to an increased risk for breast cancer in women (7, 8) and an observed decrease in human semen quality (9, 10). A model for the developmental effects of estrogen is based, to a large extent, on studies with the synthetic estrogen diethylstilbestrol (DES) in animals and humans (11–13). Most estrogenic environmental compounds have potencies 1/50th to 1/10,000th those of DES or the natural estrogen 17β-estradiol; for example, the pesticides dieldrin, toxaphene, or endosulfan have an affinity for hER that is ~1/10,000th that of estradiol (14, 15). The relatively low potencies of each of these compounds have suggested that these chemicals alone are unlikely to

Table 1. Concentrations of environmental chemicals required to achieve 50% β-Gal activity (EC_{50}) in YES. β-Galactosidase assays were done as described in Fig. 1. The EC_{50} values were generated by plotting the percent β-Gal activity versus the concentration of environmental chemical. The values for the combinations represent the total concentration of environmental chemicals as they were mixed at equal molar concentrations. The data shown are representative of at least three independent experiments. The EC_{50} values represent the concentration required to achieve half-maximal β-Gal activity for that chemical or combination of chemicals. The maximal β-Gal activity induced by the combined environmental chemicals appeared to be ~65% that of 17β-estradiol. ND, not determined.

Chemical	β-Gal EC_{50} (μM)
17β-Estradiol	0.0001
Endosulfan	>33
Dieldrin	>33
Toxaphene	>33
Chlordane	ND*
Endosulfan + dieldrin	0.092
Endosulfan + toxaphene	0.121
Endosulfan + chlordane	0.189
Dieldrin + toxaphene	0.210
Dieldrin + chlordane	0.286
Toxaphene + chlordane	0.306

*The EC_{50} for chlordane was not measured because it did not exhibit β-Gal activity at any concentration tested.

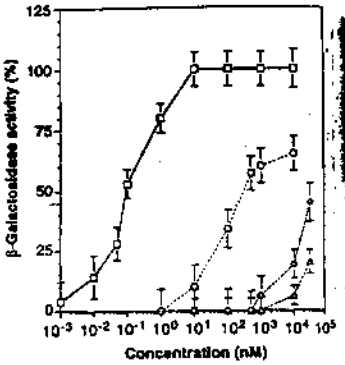
produce adverse health effects in humans (*16*, *17*). However, these compounds occur as mixtures in the environment, and their combined action has not been well studied.

To investigate the interaction of mixtures of chemicals with hER, we compared the transcriptional activation of hER in yeast in response to environmental chemicals alone and in combination (*18*). Dieldrin, endosulfan, or toxaphene alone weakly increased β-galactosidase (β-Gal) activity even at high concentrations (greater than 10 μM) (Fig. 1 and Table 1). However, a combination of any two of these chemicals produced a synergistic increase in β-Gal activity as compared with the individual compounds. For example, to increase β-Gal activity to a similar extent, the concentration of the mixture of endosulfan and dieldrin required was 1/160th to 1/1600th that of either chemical alone (that is, the mixture was 160 to 1600 times more potent than the individual chemicals). Chlordane, which had no measurable activity in the YES, also significantly enhanced the potency of the other environmental chemicals (Table 1).

Because other signaling pathways, such as those involving protein kinases activated by growth factors, play a role in estrogen action (*19*, *20*), we studied the binding of [^{3}H]17β-estradiol to hER with the environmental chemicals alone or in combination as a measure of the direct interaction of these compounds with hER (*21*). Consistent with the results in the YES, dieldrin, endosulfan, or toxaphene alone only weakly inhibited the binding of [^{3}H]17β-estradiol to hER (Fig. 2 and Table 2). To inhibit [^{3}H]17β-estradiol binding to the same extent, the concentration of the combined chemicals was at most 1/200th that required for either chemical alone (that is, the mixture was at least 200 times more potent than the individual chemicals). Chlordane, which did not inhibit binding of [^{3}H]17β-estradiol, also enhanced the competitive binding activity of the other environmental chemicals.

The synergistic action of combinations

Fig. 1. Estrogenic activity of environmental chemicals in YES. A single yeast colony was grown overnight at 30°C in synthetic media supplemented with Trp and Ura, then 50 μl was added to 950 μl of fresh media with vehicle, 17β-estradiol (□), endosulfan (◇), dieldrin (△), or endosulfan and dieldrin (○) and grown overnight at 30°C. Equivalent molar concentrations of endosulfan and dieldrin were used for the combinations (for example, 5 nM of endosulfan and dieldrin for a total of 10 nM). After treatment, the yeast cells were collected by centrifugation, the A_{600} measured, and the activity of β-Gal determined as described (*18*). The data are representative of at least three separate experiments. The increase in β-Gal activity is greater at the lower concentrations of mixtures of environmental chemicals.



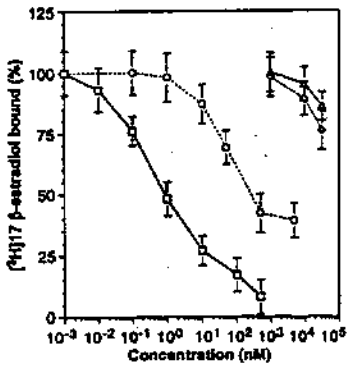


Fig. 2. Inhibition of [^{3}H]17β-estradiol binding to hER by environmental chemicals. Recombinant hER at a concentration of ~0.4 nM was dissolved in the binding buffer [10 mM tris (pH 7.4), 1 mM EDTA, 1 mM EGTA, 1 mM $NaVO_4$, 10% glycerol, γ-globulin (10 mg/ml), 0.5 mM PMSF, and 0.2 mM leupeptin] for 1 hour at 25°C with 2.5 nM[^{3}H]17β-estradiol in the absence or presence of the indicated concentrations of radioinert environmental chemicals or 17β-estradiol. Equivalent molar concentrations of endosulfan and dieldrin were used for the combination. Free [^{3}H]17β-estradiol was removed by incubation with Chardex for 10 min at 4°C and centrifugation for 3 min at 15,000*g*. The data shown are representative of at least three independent experiments. The decrease in [^{3}H]17β-estradiol binding is greater at the lower concentrations of mixtures of environmental chemicals. Symbols as in Fig. 1.

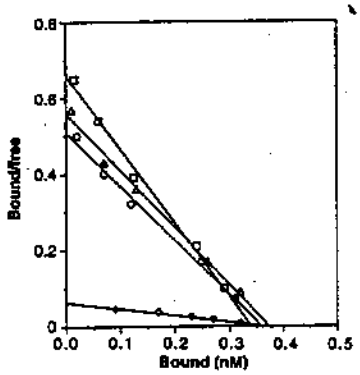


Fig. 3. A Scatchard analysis of hER binding to [^{3}H]17β-estradiol in the absence or presence of environmental chemicals. Recombinant hER was dissolved in binding buffer for 1 hour at 25°C (see Fig. 1) with 0.5, 1, 2.5, 5 or 10 nM [^{3}H]17β-estradiol and a 300-fold molar excess of unlabeled 17 β-estradiol (□), 100 nM endosulfan (○), 100 nM dieldrin (△), or 100 nM endosulfan and 100 nM dieldrin (◇). The data were analyzed by the method of Scatchard (*31*) and are representative of at least three independent experiments.

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assessed by addition of 300-fold molar excess of radioinert 17β-estradiol. Free [^{3}H]17β-estradiol was removed by incubation with Chardex (contains 5% activated charcoal and 0.5% dextran dissolved in phosphate-buffered saline) for 10 min at 4°C and centrifugation for 3 min at 15,000*g*. The bound [^{3}H]17β-estradiol was measured by scintillation counting. The data shown are representative of at least three independent experiments (D. M. Klotz, J. A. McLachlan, S. F. Arnold, in preparation).

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32. Ishikawa cells grown in a 35-mm plate were transfected with 2 μg of pERE-luc (containing two copies of the vitellogenin estrogen response element linked to the luciferase gene), 1 μg of a control β-Gal plasmid, and 20 ng of hER in a mammalian expression vector with 10 μl of lipofectamine (GIBCO BRL) for 5 hours. The cells were treated with vehicle, estradiol, or 2′,4′,6′-trichloro-4-biphenylol or 2′,3′,4′,5′-tetrachloro-4-biphenylol (or both) in Dulbecco's modified Eagle's medium with 5% charcoal-stripped fetal bovine serum for 18 hours. All chemicals were dissolved in DMSO and added to the media so that the concentration of DMSO did not exceed 1%. The cells were collected by incubation with lysis buffer (Analytical Luminescence, Ann Arbor, MI) for 15 min at 25°C, the protein concentration determined with the Bio-Rad protein assay reagent, and the β-Gal activity measured as described (*18*). The amount of extract used in the luciferase assay was normalized for protein and β-Gal activity. The luciferase assay was performed in the Monolight 2010 as recommended by the manufacturer (Analytical Luminescence).

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Regulation of the Inositol 1,4,5-Trisphosphate Receptor by Tyrosine Phosphorylation

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Tyrosine kinases indirectly raise intracellular calcium concentration ($[Ca^{2+}]_i$) by activating phospholipases that generate inositol 1,4,5-trisphosphate (IP_3). IP_3 activates the IP_3 receptor (IP_3R), an intracellular calcium release channel on the endoplasmic reticulum. T cell receptor stimulation triggered a physical association between the nonreceptor protein tyrosine kinase Fyn and the IP_3R, which induced tyrosine phosphorylation of the IP_3R. Fyn activated an IP_3-gated calcium channel in vitro, and tyrosine phosphorylation of the IP_3R during T cell activation was reduced in thymocytes from *fyn*$^{-/-}$ mice. Thus, activation of the IP_3R by tyrosine phosphorylation may play a role in regulating $[Ca^{2+}]_i$.

The IP_3R forms the IP_3-gated Ca^{2+} release channel on the endoplasmic reticulum in many cell types, including neurons (*1*) and T cells (*2*). The human type 1 IP_3R (IP_3R1) is a 308-kD polypeptide that contains two potential tyrosine phosphorylation sites, at residues 482 (Glu-Asp-Leu-Val-Tyr) and 2617 (Asp-Ser-Thr-Glu-Tyr) (*2*). Amino acid 482 is adjacent to the IP_3-binding site (*3*), and amino acid 2617 is near the COOH-terminus (*4*).

The IP_3R is autophosphorylated on serine and is phosphorylated by protein kinases A, C, and G and by Ca^{2+}-calmodulin–dependent kinase II (CaMkII) in vitro (*5*). Members of the Src family of nonreceptor protein tyrosine kinases function in signal transduction in the brain (*6*) as well as in T cells (*7*, *8*). To determine whether tyrosine phosphorylation of the IP_3R occurred, we immunoprecipitated the Ca^{2+} release channel with an IP_3R1-specific antibody (anti-IP_3R1) from both brain and T lymphocytes and subjected it to kinase assays with two nonreceptor protein tyrosine kinases, Src and Fyn. Both kinases induced tyrosine phosphorylation of the brain IP_3R (Fig. 1A). Similar results were obtained with canine, rabbit, and murine brain microsomes (*9*) and with purified IP_3R (Fig. 1A), which suggests that the kinase acts on the channel directly, not through an intermediary molecule.

Fyn and Src induced tyrosine phosphorylation of the IP_3R from unstimulated Jurkat cells (Fig. 1B) in vitro. Tyrosine phosphorylation of the IP_3R from unactivated Jurkat cells by Fyn was greater than that of the IP_3R from cells activated by incubation with monoclonal antibody to CD3 (CD3 mAb) (Fig. 1B). This result indicated that Fyn induces tyrosine phosphorylation of the IP_3R during T cell receptor (TCR) stimulation, and therefore that exogenous Fyn does not induce further tyrosine phosphorylation of the IP_3R in vitro.

To determine whether tyrosine phosphorylation of the IP_3R occurred in vivo, we prepared anti-phosphotyrosine immunoblots of IP_3R immunoprecipitated from unstimulated and from TCR-stimulated Jurkat cells. IP_3R was detected in both activated and unactivated cells, but tyrosine-phosphorylated IP_3R was detected only in TCR-stimulated cells (Fig. 1C). This finding indicated that tyrosine phosphorylation of the IP_3R occurs in intact cells.

During T cell activation, Fyn physically associates with the TCR (*10*) and the IP_3R cocaps with the TCR (*11*). To determine whether Fyn physically associated with the IP_3R during T cell activation, we used anti-IP_3R1 or Fyn mAb to immuno-

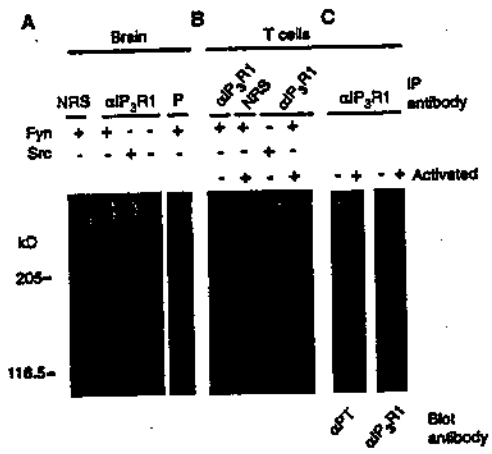

Fig. 1. Tyrosine phosphorylation of the ~300-kD IP_3R in brain and T lymphocytes (Jurkat) by Src and Fyn. IP_3R was immunoprecipitated from canine brain microsomes (A) and from lysates of unactivated Jurkat cells or cells activated with CD3 mAb (B). Immunoprecipitated proteins or purified IP_3R (P) were used in kinase assays with or without exogenous Fyn or Src (*21*). Preimmune serum (NRS) or affinity-purified anti-IP_3R1 (αIP_3R1) were used for immunoprecipitations (*21*). (C) Immunoprecipitated IP_3R from activated or unactivated Jurkat cells was immunoblotted with anti-phosphotyrosine (αPT) (4G10) and then, after stripping, with anti-IP_3R1 as described (*2*).

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of environmental chemicals suggested the possibility of additional binding sites on hER (22–24). Linear slopes observed in Scatchard analyses of binding experiments with combined environmental chemicals demonstrated competitive inhibition (Fig. 3), suggesting that the chemicals interact in combination with hER at the hormone binding site (25).

Hydroxlyated polychlorinated biphenyls (PCBs) activated estrogen-dependent reporter activity in the YES and in Ishikawa cells, an endometrial cancer cell line transiently transfected with hER, and an estrogen-sensitive luciferase reporter (Table 3). A mixture of the two PCBs synergistically activated reporter gene activity in the YES and in Ishikawa cells. In vitro binding experiments confirmed that PCBs interacted with the hER and when mixed displayed a synergistic reduction in [^{3}H]17β-estradiol binding to hER. Compound F demonstrated a median inhibitory concentration (IC_{50}) of 55 nM, whereas compound G had an IC_{50} of 120 nM; both compounds added together resulted in an IC_{50} of 5 nM.

Our results provide a molecular explanation for studies in vivo that described synergistic effects of environmental estrogens. For example, the addition of weakly estrogenic PCB congeners to developing turtle eggs resulted in sex reversal of male-determined eggs; addition of two PCBs at subthreshold concentrations were effective (26). Likewise, dieldrin, endosulfan, or toxaphene, although only marginally effective in stimulating proliferation of human breast cancer cells in culture, exhibited greater than additive effects when administered together (15). Apparently synergistic estrogenic effects of environmental chemicals were observed in cultures of fish hepatocytes (27). Our data and the above reports suggest that the estrogenic potency of some environmental chemicals, when tested singly, may be underestimated.

In addition to their environmental implications, these findings may also have general biological significance, because ovarian and phytoestrogens act synergistically in the YES (28). Transciptional activation of hER through interaction with two chemicals is consistent with the observation that the active site of a cytochrome P_{450} has the potential to recognize two substrates (29). The possibility for synergistic action of apparently inactive chemicals functioning as hormones may represent a previously uncharacterized level of receptor-mediated gene regulation. The interaction of multiple chemicals with the estrogen receptor suggests a complex interplay between environmental signals and biological systems.

Table 2. Concentrations of environmental chemicals required to achieve 50% displacement or inhibition (IC_{50}) of hER binding of [^{3}H]17β-estradiol. Competitive binding assays were done as described (Fig. 2). The IC_{50} values were calculated by plotting the percent [^{3}H]17β-estradiol remaining versus the concentration of environmental chemical as shown in Fig. 2. The values for the combinations represent the total concentration of environmental chemicals as they were mixed at equal molar concentrations. The data shown are representative of at least three independent experiments. The standard error was less than 7.5% for the competition binding assays.

Chemical	hER binding IC_{50} (μM)
Endosulfan	>50
Dieldrin	>50
Toxaphene	>50
Chlordane	ND*
17β-Estradiol	0.001
Endosulfan + dieldrin	0.324
Endosulfan + toxaphene	0.339
Endosulfan + chlordane	0.363
Dieldrin + toxaphene	0.498
Dieldrin + chlordane	0.514
Toxaphene + chlordane	0.533

*The IC_{50} value was not determined because chlordane did not appear to demonstrate competitive binding activity at any concentration tested. It has been reported that the IC_{50} values for endosulfane and toxaphene are 631 and 470 μM, respectively (15).

Table 3. Concentrations of environmental chemicals required to achieve 50% β-Gal activity in YES (18) or luciferase activity in Ishikawa cells (32). The PCB compounds included F (2′,4′,6′-trichloro-4-biphenylol) and G (2′,3′,4′,5′-tetrachloro-4-biphenylol). The difference in the EC_{50} values between the β-Gal and luciferase assays is probably due in part to a higher concentration of hER in Ishikawa cells compared with the YES. The EC_{50} values were generated by plotting the percent β-Gal or luciferase activity versus the concentration of environmental chemical. The values for the combinations represent the total concentration of environmental chemicals as they were mixed at equal molar concentrations. The data shown are representative of at least two independent experiments. The standard error was less than 7.5% for the assays.

Chemical	β-Gal EC_{50} (μM)	Luciferase EC_{50} (μM)
Estradiol	0.0001	0.0002
F	0.0070	0.0120
G	0.0180	0.0300
F + G	0.0015	0.0025

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18. YES was developed by transforming yeast strain BJ2407 (*Mata/Matα ura3-52, trp1, leu2, srb1-1122, prc1-407, pep4-3*) with an expression plasmid containing the complementary DNA of hER and a reporter plasmid containing two estrogen response elements linked to the *lacZ* gene. A single yeast colony was grown overnight at 30°C in synthetic media supplemented with Trp, Ura, then 50 μl was added to 950 μl of fresh media with vehicle, 17β-estradiol, endosulfan, dieldrin, or endosulfan and dieldrin and grown overnight at 30°C. The environmental chemicals and estradiol were dissolved in dimethyl sulfoxide (DMSO), and the concentration of DMSO in the media did not exceed 1%. The solubility limit for the environmental chemicals was between 33 and 50 μM, and therefore concentrations greater than 33 μM were not used. After treatment, the yeast cells were collected by centrifugation and permeabilized by the addition of 700 μl of Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 35 mM β-mercaptoethanol), 4 μl of 0.1% SDS, 6 μl of $CHCl_3$ and mixed for 25 s. The reactions were then placed at 30°C with the addition of 160 μl of *o*-nitrophenyl β-D-galactopyranoside (4 mg/ml in Z-buffer). The reactions were terminated by the addition of 400 μl of 1 M $NaCO_3$, and the absorbance at 420 nm (A_{420}) was measured. β-Galactosidase activity was determined by the formula $[A_{420}/(A_{600}$ of 1/10 dilution of cells × volume of culture × length of incubation)] × 1000 and expressed as a percentage of the maximal response to estradiol (S. F. Arnold, M. K. Robinson, A. C. Notides, L. J. Guillette Jr., J. A. McLachlan, *Environ. Health Perspect.* 104, 544 (1996).
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21. We produced recombinant hER in Sf9 insect cells by using the baculovirus expression system and prepared the hER as ammonium sulfate precipitates. The hER produced in Sf9 cells has approximately the same affinity for estradiol as hER produced in mammalian and yeast cells (30). Recombinant hER at a concentration of ~0.4 nM was dissolved in the binding buffer [10 mM tris (pH 7.4), 1 mM EDTA, 1 mM EGTA, 1 mM $NaVO_4$, 10% glycerol, γ-globulin (10 mg/ml), 0.5 mM phenylmethylsulfonyl fluoride (PMSF), and 0.2 mM leupeptin for 1 hour at 25°C with 2.5 nM [^{3}H]17β-estradiol in the absence or presence of increasing concentrations of radioinert environmental chemicals or 17β-estradiol. [^{3}H]17β-estradiol, endosulfan, and dieldrin were dissolved in DMSO at 10 mM and added to the reaction so that the concentration of DMSO did not exceed 2.5%. Equivalent molar concentrations of endosulfan and dieldrin were used for the combination. Endosulfan or dieldrin were not tested at concentrations greater than 33 μM. Nonspecific binding of [^{3}H]17β-estradiol was

BRENDA HOELMAN
ATTACHMENT #8

ENVIRONMENTAL ESTROGENS

New Yeast Study Finds Strength in Numbers

The notion that modern industrial society is producing hormonelike pollutants that can interfere with human reproduction has become a hot topic in the media and within Congress in recent months. A widely promoted book, *Our Stolen Future* (see review on p. 1444), with a preface by Vice President Al Gore, put the theory high on the public agenda—and drew a strong response from some researchers who pointed out that the pollutants don't have nearly the clout of natural estrogens in the body and, thus, may have no significant impact on humans. Now, a paper in this issue (p. 1489; also see Perspective on p. 1451) is likely to add fresh fuel to the debate. A team of researchers from Tulane University in New Orleans, using a novel screening system based on genetically engineered yeast cells, reports that a mixture of two weakly estrogenic chemicals can be far more potent than the individual compounds.

Strong synergy. Tulane's Collins, Klotz, McLachlan, and Arnold test combinations.

The findings are causing scientists to take a fresh look at the controversy. "It's a very striking result," says Wade Welshons, an endocrinologist at the University of Missouri. "It doesn't forge a direct connection between developmental estrogen problems and these chemicals, but it's a very important red flag." Others caution, however, that more work must be done to pin down whether the mechanism found in yeast cells has any relevance to humans. "These are very interesting observations, but they raise more questions than they answer," says Jack Gorski, a biochemical endocrinologist at the University of Wisconsin.

The Tulane research addresses one of the hottest controversies in toxicology: Do estrogenlike compounds in the environment—for example, pesticides, the plastics ingredient bisphenol-A, and some polychlorinated biphenyls (PCBs)—contribute to such ills as breast cancer, a possible drop in human sperm counts, and a rise in testicular cancer (*Science*, 15 July 1994, p. 308)? Some researchers have linked spills of such chemicals with reproductive abnormalities in wildlife, but the debate centers on whether the low levels present in the environment are sufficient to harm humans.

Two years ago, Tulane environmental endocrinologist John McLachlan, then scientific director of the National Institute of Environmental Health Sciences, and collaborators came upon a possible clue. The group was able to make male turtle embryos develop into females by painting the eggs with estradiol—the body's main estrogen—or certain estrogenic PCBs. At moderate doses they achieved this effect only when they combined two PCB compounds; the same PCBs applied individually were ineffective. So 6 months ago, when McLachlan teamed with molecular endocrinologist Steve Arnold and University of Florida reproductive physiologist Louis Guillette to set up a yeast system to screen for environmental estrogens, they decided to test various mixtures of the compounds.

The system consists of yeast cells engineered to contain genes that code for the human estrogen receptor and a "reporter" protein that the cell makes when an estrogenlike compound binds to the receptor. The culture turns blue when a chemical binds to the receptor, and the intensity of the color reflects how strongly the receptor is activated.

Tests on four pesticides believed to be only very weakly estrogenic—the pesticides dieldrin, endosulfan, toxaphene, and chlordane—yielded little or no response, as expected. (All but endosulfan have been banned in the United States, but they persist in the environment, sometimes in combination.) When the chemicals were paired, however, the activity shot up by a factor of 160 to 1600. "It was really quite astounding," McLachlan says. The group also found a fivefold synergistic effect in the yeast cells with a PCB mixture that had reversed the sex of the turtle eggs. And they showed that their results were not specific to the yeast system by getting comparable effects with PCBs in human endometrial cells.

Although the various combinations of pesticides were only 1/500 to 1/15 as potent as estradiol itself, McLachlan says his group worked with "levels [of environmental estrogens] actually achieved in some systems," such as the turtle eggs and PCBs in the serum of a group of women with breast cancer. The results, he says, "at least provide a mechanism where low levels of weak-acting environmental estrogens could have a greater-than-expected effect."

Other researchers emphasize that the results must be verified in various animal species to establish whether they are relevant to wildlife or people. The yeast-cell system "is a good controlled experimental system. But these are the first observations from the system," says toxicologist Michael Gallo of the Robert Wood Johnson Medical School in New Jersey. "[Now researchers] have to move into different phyla and ratchet down on the molecular explanation." McLachlan's team is now studying the estrogen receptor's binding pockets in search of a molecular mechanism.

Toxicologist Stephen Safe of Texas A&M University, a vocal skeptic of the notion that environmental estrogens are linked to human health effects, agrees that the findings "are really interesting and may have environmental significance." But he says the data do nothing to undermine a major criticism of the hypothesis: that many synthetic and natural environmental estrogens, including some in plants, are actually "antiestrogenic"—they block or reduce the activity of estrogen receptors—and could cancel out even powerful synergistic estrogenic effects. "We have to look at the opposite side of the coin," Safe says. McLachlan acknowledges this possibility and says his group has begun testing antiestrogenic chemicals and estrogenic/antiestrogenic combinations.

For now, the findings will stimulate more studies of chemical cocktails—an area largely overlooked in recent research on endocrine disrupters, which has focused on individual compounds. And if the results do hold up in various animal species, scientists may need to revise their current assumption that the effects are additive. "The safety margin may be a lot smaller than has been anticipated," says toxicologist John Gierthy of the New York State Department of Health. It could also "make testing extremely complex," he adds.

Indeed, the results may need to be taken into account by an Environmental Protection Agency (EPA) advisory panel now being formed to come up with in vitro test strategies to screen for environmental estrogens that pose the greatest potential threat, says Lynn Goldman, head of the EPA Office of Prevention, Pesticides, and Toxic Substances. Legislation pending in Congress would require EPA to begin screening such chemicals within 2 years. The Tulane findings could have "enormous policy implications" for EPA, says Goldman. "Obviously," she says, "these systems are more complex than we had imagined."

—Jocelyn Kaiser

ATTACHMENT #9

New multiple sclerosis drug clears hurdle

Multiple sclerosis (MS) begins as a tragic case of mistaken identity.

In this autoimmune disease, white blood cells, the body's roving guardians against infection, view the fatty sheath surrounding nerve fibers as a threat. The cells promptly begin to digest bits of the nerves' insulating coating. Like frayed wires, the damaged nerves short-circuit, blocking communication between brain and muscles.

Fascinated with such processes, Ruth Arnon, Michael Sela, and Dvora Teitelbaum at the Weizmann Institute of Science in Rehovot, Israel, set out 27 years ago to create synthetic molecules capable of provoking an immune response. They ended up developing an entirely new way of treating MS—and perhaps other autoimmune diseases as well.

The pivotal step involved making a replica of a protein from the sheath of human nerve cells. This protein triggers an MS-like response when injected into guinea pigs, and the researchers had hoped that their replica would do the same. Instead, they found, it appears to act as a decoy, diverting the immune onslaught from nerve tissue.

"It is a new approach to the treatment of autoimmune diseases," Sela asserts. Although it will not prevent the disease, he says he thinks of the new treatment as "a synthetic 'vaccine' against MS."

The research achieved a practical milestone on Sept. 19. A Food and Drug Administration advisory committee recommended that the agency approve one of the group's decoys, copolymer 1, as a treatment for MS. The FDA typically follows its advisory committees' advice.

If approved, copolymer 1 would be the third drug in 3 years okayed for the treatment of MS, an incurable disease known for intermittent and progressively more severe episodes of pain and paralysis.

Until recently, all doctors could do to slow the steady slide into paraplegia was prescribe steroids. They hoped that the anti-inflammatory drugs would hamper the immune system, forcing it to leave the nerves alone. That solution rarely worked for long.

The two other new remedies for MS are Avonex and Betaseron, genetically engineered versions of the immune modulator interferon. Both cause unpleasant, flulike side effects.

Copolymer 1, which patients must inject daily, appears to be free of major side effects other than a temporary inflammation at the injection site and a fleeting tightness in the chest. Like the interferons, copolymer 1 cuts the number of MS episodes by about one-third.

"Two well-controlled, double-blind studies indicate that the drug reduces the rate of relapses of MS," says Sid Gilman, chairman of the FDA advisory committee and a neurologist at the University of Michigan Medical Center in Ann Arbor.

Made of a combination of four amino acids, copolymer 1 was developed for clinical use by Israeli chemical giant Teva Pharmaceutical Industries. The company trade-named the new drug Copaxone and designed the larger of the two trials that demonstrated its effectiveness.

This study, carried out at 11 medical centers in the United States, involved approximately 250 patients. Half of them received the drug; the other half were given a placebo.

Completed in 1994, the study found that people taking copolymer 1 for 35 months had 32 percent fewer relapses than those taking the placebo. People in the drug treatment group were also more likely to improve or retain the nerve function they had when entering the study, whereas those taking the placebo tended to get worse, says Teva Vice-President Carole Ben-Maimon, who presented the company's case to the FDA committee. An earlier study of just 50 patients yielded similar results, she says.

After the committee's vote, Ben-Maimon expressed delight that the members were convinced by the firm's evidence. "We're happy for patients as well as ourselves." — *S. Sternberg*

*

'Clean' water may infect swimmers

[illegible]

ATTACHMENT #10

Aquatic Toxicology, 27 (1993) 361–372

AQTOX 00602

Detergent components in sewage effluent are weakly oestrogenic to fish: An in vitro study using rainbow trout (*Oncorhynchus mykiss*) hepatocytes

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Alkylphenol-polyethoxylates (APnEO, $n = 1$–40) are a major group of surfactants and are normally present in raw sewage. Many of the products of the biodegradation of these compounds are both persistent and present in substantial quantities in effluent and in river water. We report here on the use of an in vitro bioassay to determine the oestrogenic potencies of these compounds to fish. The bioassay is based on the fact that the synthesis of vitellogenin by hepatocytes is oestrogen dependent. Of the compounds tested, 4-nonylphenol, 4-*tert*-octylphenol, 4-*tert*-butylphenol, 4-nonylphenol-diethoxylate, Tergitol-NP9, and 4-nonylphenoxycarboxylic acid were all weakly oestrogenic, with potencies between about 1×10^{-4} to 1×10^{-6} the activity of 17β-oestradiol The oestrogenic activity observed appeared to be confined to *para* or 4 substituted compounds, because 2-*tert*-butylphenol and 3-*tert*-butylphenol were inactive. The polyethoxylate compounds became less oestrogenic with increasing lengh of the ethoxy chain. Thus cells exposed to Tergitol NP40EO (with a chain length of 40) did not secrete vitellogenin. Simultaneous exposure of the hepatocytes to Tamoxifen (an oestrogen antagonist) and effective doses of representative compounds caused an inhibition of the oestrogenic effect in all cases, suggesting that the action of these compounds is mediated by the oestradiol receptor.

Key words: Oestrogenic; Vitellogenin; Alkylphenol; *Oncorhynchus mykiss*; Sewage; Hepatocyte

INTRODUCTION

A large number of man-made chemicals are present in the environment as pollutants and are capable of disrupting the endocrine system of animals, including fish and humans. Notable amongst such chemicals are those which act as oestrogens. In vertebrates, the primary natural oestrogen is 17β-oestradiol, which has many important roles, including stimulating the growth and development of the female sex or-

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gans. A very wide range of substances have been shown to mimic the action of 17β-oestradiol, including synthetic steroids such as those used in the contraceptive pill (Pelissero et al., 1993), many pesticides (Chambers, 1984; Eroschenko, 1981; Johnson et al., 1992) and phytoestrogens (Pelissero et al., 1989, 1991).

It was recently reported (Soto et al., 1991) that another group of chemicals, the alkylphenols (AP), are weakly oestrogenic in mammals. These compounds are the final products of the biodegradation of a family of non-ionic surfactants (alkylphenol-polyethoxylates or APnEO, n = 9–40) during sewage treatment (Giger et al., 1984, 1987). Annual usage of nonylphenol-polyethoxylates in Europe is around 75,000 tonnes. During sewage treatment, the APnEOs are biodegraded first into APnEOs with shorter ethoxylate chains and finally into alkylphenols themselves. The major pathways of this slow degradation process have been previously demonstrated (Giger et al., 1987) and are summarised in Figure 1. The intermediates illustrated in Figure 1 are present in sewage effluent and also in river water throughout Europe and the USA (Ahel and Giger, 1985; Garrison and Hill, 1972; Giger et al., 1984; Marcomini et al., 1990; Stephanou and Giger, 1982; Thoumelin, 1991; Wahlberg et al., 1990). The concentrations that have been reported are extremely variable and range from nanograms to milligrams/litre; the most recent and comprehensive study is reported in Naylor et al. (1992). In addition, Clark et al. (1992) have reported the presence of both APnEOs and their carboxylic acid derivatives (APnEC, n = 0–3) in drinking water.

Because of the relatively high concentrations of these compounds sometimes found in effluents and in river water, we decided to extend the studies of Soto et al. (1991) by determining whether the alkylphenols (AP), found to be oestrogenic in mammals, are also oestrogenic in fish and further, to assess whether the APnEOs and the APnECs (found in the effluent and in river water in greater quantities than the APs) are also active. Our studies were conducted using an in vitro bioassay which is based on the ability of primary cultures of rainbow trout (*O. mykiss*) hepatocytes to respond to oestrogenic stimulation by producing vitellogenin (Pelissero et al., 1993). Vitellogenin is a large lipoglycophosphoprotein which is normally secreted by the liver of female fish in response to endogenous oestrogens (Chen, 1983) and is carried by the blood to the ovaries, where it is selectively sequestered by the developing oocytes (Tyler et al., 1988a,b). Here it forms the major constituent of yolk, which is subsequently utilised by the growing embryo as a food source (for reviews see Wallace, 1985 and Tyler, 1991).

MATERIALS AND METHODS

Fish

The fish used were all male rainbow trout and were collected from a local trout farm (Reading, Berkshire, UK). Their average weight was 300 g. They were mostly immature, although some were maturing.

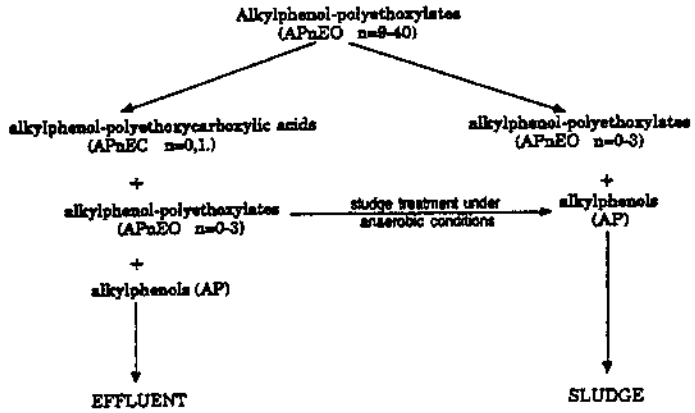

Fig. 1. The biodegradation of surfactants and detergents during sewage treatment (based on the work of Giger et al., 1987). AP is an alkylphenol. The alkyl chain is of the form C_nH_{n+1}. APnEO can refer to any alkylphenol-polyethoxylate (nonyl, octyl or butyl).

Perfusion of liver and preparation of cells

The perfusion was performed according to Pelissero et al. (1993) using a two-step method. The essential feature of this procedure is that the liver is first flushed with a calcium-free medium (solution 1) and then with one containing calcium and collagenase (solution 2). Each fish was killed by a blow to the head and cleaned with alcohol. Under sterile conditions, 5,000 U of Heparin (Sigma, Dorset, UK) was injected into the heart to prevent the blood clotting. The liver was perfused at a rate of 10 ml/min via the intestinal artery. The heart was severed at the onset of the perfusion to avoid excess pressure in the blood system. Solution 1 contained 160 mM NaCl, 10 mM HEPES, 3 mM KCl and 0.3 mM Na_2HPO_4 (pH 7.8). This was perfused for 20–30 min to facilitate blood removal and the cleavage of hepatic desmosomes. Solution 2 contained 140 mM NaCl, 8 mM HEPES, 3 mM KCl, 10 mM $CaCl_2$ and 260 mg collagenase/l. The collagenase was grade H (Boehringer Mannheim, East Sussex, UK), and the amount used was equivalent to 39 U/l. The duration of perfusion with solution 2 was determined by the size of the liver, but was generally for 15 to 30 min. The liver was then removed and torn into small pieces in a sterile petri dish containing 20 ml of 'phenol red-free' Dulbecco's Modified Eagle's Medium supplemented with Ham's F12 nutrients (DMEM + Ham's F12; Sigma, Dorset, UK), buffered with 13 mM HEPES, 15 mM TES and containing 20 ml Ultroser SF (steroid free) serum (Jones Chromatography, Mid Glamorgan, UK), 5 ml Fungizone (250 μg/ml; Gibco, Middlesex, UK) and 2 ml of each of the following antibiotics: Antibiotic antimycotic mixture (10 U Penicillin, 10 mg Streptomycin, 25 μg Amphotericin/ml solution; Sigma, Dorset, UK), Gentamicin (10 mg/ml; Sigma, Dorset, UK) per litre of medium. The pH of the medium was adjusted to 7.8 with 0.5 M NaOH before sterile filtration.

The resulting cell suspension was filtered through a sterile pad (200 μm pore diameter) and centrifuged at 30 × *g* for 1 min at room temperature. After aspiration of the supernatant, the cells were resuspended in fresh medium and re-centrifuged. This washing step was repeated until the supernatant was clear. A concentrated cell suspension was prepared and cell viability was determined using Trypan blue dye exclusion. Finally, a suspension of 1,000,000 viable cells/ml was prepared and 3 ml of this suspension were added to each well.

Culture conditions

Freshly prepared hepatocytes were distributed into 30 mm diameter wells and maintained in 'phenol red-free' medium (DMEM + Hams F12, as above). Every 48 h throughout the entire culture, 2 ml of medium was removed from each well and replaced with new medium. The cells were shaken gently throughout on an orbital shaker. This method of incubating the cells followed the method of Pelissero et al. (1993) and in 2 to 4 days leads to the formation of aggregations of cells in the centre of each well. Once the aggregates were formed, each of the test solutions was added to three replicate wells (100 μl of test solution, 1.9 ml of fresh medium/well). 100 μl of 0.3% ethanol in medium were administered to the control wells. Subsequently, after an incubation period of a further 2 days, the medium from each of the wells was collected and stored at −20°C until required for assay. Greater secretion of vitellogenin by the cells could be obtained by restimulation and incubation for a further 2 days, before collecting the medium for assay.

Compounds tested

Table 1 lists the compounds tested, the shorthand notation to which they are re-

TABLE 1
List of compounds tested, shorthand notation used in text and origin

Compound	Abbreviation	Origin
4-nonylphenol	4-NP	MTM, Lancashire, UK
4-*tert*-butylphenol*	4-tBP	Aldrich, Dorset, UK
4-*tert*-octylphenol*	4-tOP	Aldrich, Dorset, UK
2-*tert*-butylphenol*	2-tBP	Aldrich, Dorset, UK
3-*tert*-butylphenol*	3-tBP	Aldrich, Dorset, UK
4-nonylphenoldiethoxylate	NP2EO	ICI, Cleveland, UK
Tergitol NP9	NP9EO	Sigma, Dorset, UK
Tergitol NP40	NP40EO	Sigma, Dorset, UK
4-nonylphenoxycarboxylic acid	NP1EC	Aldrich, Dorset, UK
17β-Oestradiol	None	Sigma, Dorset, UK
Tamoxifen	None	Sigma, Dorset, UK

*'*tert*' is the accepted abbreviation for tertiary.

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TABLE 2
Relative oestrogeni

Compound
17β-oestradiol
NP
4-tBP
4-tOP
NP2EO
NP9EO
NP1EC

[a]Refers to the numb
[b]Refers to the mean

ferred to in the text, and their origin. Most of the compounds tested were not highly soluble in water. All of the compounds were therefore dissolved first in ethanol. 1 mM stock solutions were prepared, after which serial dilutions in medium were carried out in order to achieve final concentrations from 100 nM to 100 μM (for each of the xenobiotics), and 1 pM to 1 μM (for oestradiol-17β). The concentration of ethanol in the wells was 0.3% throughout.

Vitellogenin assay

Vitellogenin concentrations in each of the wells were determined at the beginning and at the end of each experiment by radioimmunoassay as described previously (Sumpter, 1985).

Statistics

Sigmoidal dose–response curves were fitted to the data obtained for each of the compounds tested by non-linear regression using a STATSVIEW computer program (Apple Macintosh, USA). ED50s were calculated and averaged for each compound over several experiments. Using the linear portion of each curve, parallel line analyses were used to compare each compound in turn to oestradiol. The slopes of each pair of regression lines were compared using a covariance analysis (Scherer, 1984), and in cases where no significant differences ($P > 0.05$) were observed, relative potencies throughout a particular range could be calculated. These were assessed by comparing the various concentrations at which the secretion of vitellogenin due to each compound was equal to that due to 17β-oestradiol. Ratios were obtained (concentration of 17β-oestradiol/concentration of compound), and the means of these values are given in Table 2. In cases of non-parallelism, the ED50s of each curve were used to calculate relative potencies at this dose only. Using an SPSSX statistical computing package, one-way analysis of variance was used to determine differences in the mag-

TABLE 2
Relative oestrogenic potencies of the active compounds

Compound	N[a]	Mean ED 50	Standard error	RP[b]
17β-oestradiol	8	1.81 nM	0.81	1
NP	4	16.15 μM	0.79	0.0000090
4-tBP	3	2.06 μM	0.57	0.0001600
4-tOP	2	2.11 μM	0.22	0.0000370
NP2EO	2	17.27 μM	0.77	0.0000060
NP9EO	2	82.31 μM	7.79	0.0000002
NP1EC	2	15.25 μM	2.76	0.0000063

[a]Refers to the number of experiments conducted.
[b]Refers to the mean potency of each compound relative to 17β-oestradiol.

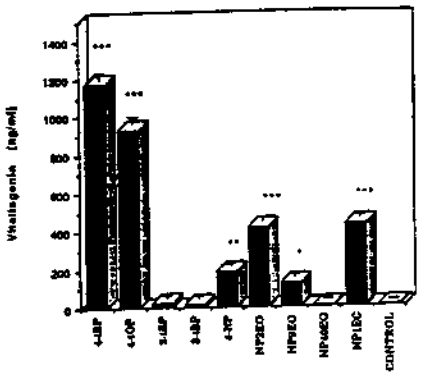
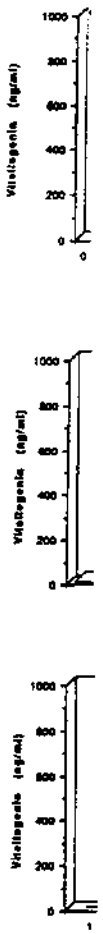


Fig. 2. Vitellogenin production from cultured hepatocytes after 4 days of exposure to 10 μM of each of the test compounds. Each column represents the mean of five or six replicate observations, vertical bars show the standard error. $^{*}P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.001$ compared with the control. NP1EC and NP2EO are equally potent, as are NP and NP9EO. NP40, 2-tBP and 3tBP values are not significantly different from the control ($P > 0.05$). Control values are below the detection limit of the assay (10 ng/ml).

nitude of the response with different compounds. This was followed by Scheffes test for multiple comparisons.

RESULTS

Figure 2 compares the magnitude of response for each of the compounds measured, after 4 days exposure, at a single concentration of 10 μM. These results were obtained using a bioassay with six replicate wells for each compound. There was probably no synthesis of vitellogenin in the control wells, as no vitellogenin was detectable in the medium (the detection limit of the assay was 10 ng/ml). Conversely, there were very marked differences in the activities of the various test compounds. The most active was 4-tBP, which enhanced vitellogenin synthesis markedly, so that the concentration in the medium was more than 100-fold above the control value. 4-tOP was almost as potent as 4-tBP; it caused at least a 90-fold increase in the amount of vitellogenin secreted ($P < 0.001$). The response induced by 4-tBP was significantly higher than those observed with each of the other compounds ($P < 0.05$), which in turn were significantly different from one another. Exceptions were NP2EO and NP1EC, and 4-NP and NP9EO, which were of equal potency. The concentrations of vitellogenin which were measured in wells containing the former two compounds were 40–50-times greater than that of the control ($P < 0.001$), whilst those containing the latter caused an 18–20-fold increase. The concentration of vitellogenin found in wells containing NP40EO, 2-tBP and 3-tBP was not significantly different from that found in the control wells ($P > 0.05$).



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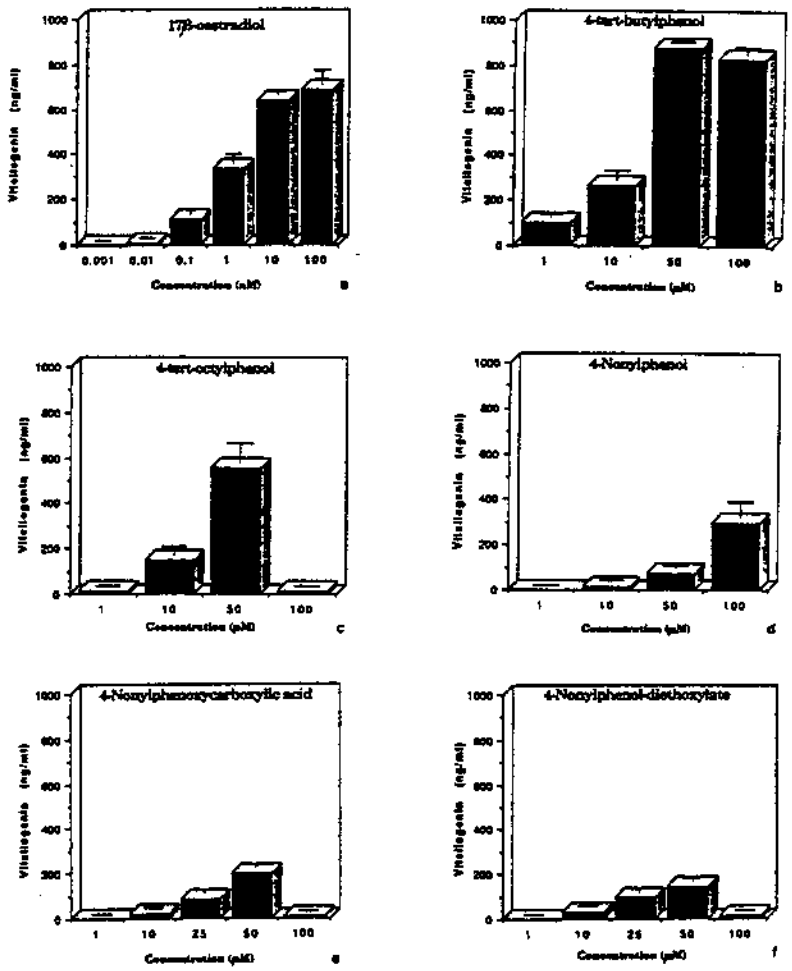

Fig. 3. Dose–response curves for each of the active test compounds after 2 days exposure. The production of vitellogenin is dose related and decreases at higher doses due to the toxicity of the compounds under test. Values shown are the means of triplicate observations and the error bars represent the standard error.

Figure 3 demonstrates by use of examples that the secretion of vitellogenin observed in this study was dose related. After a 2-day exposure to each of the active compounds, dose–response curves could be obtained. Using 17β-oestradiol, the minimum concentration required to induce a significant response was 10 pM and a maximum response occurred around 100 nM (Fig. 3a). Of the xenobiotics tested (Fig. 3b–f), those which were found to be potent were generally active in the micromolar

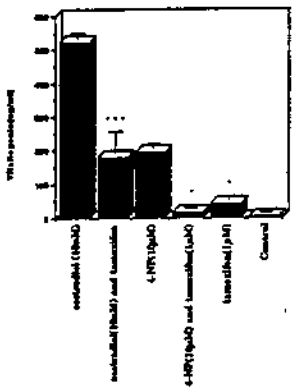

Fig. 4. Effect of Tamoxifen (1 μM) on the production of vitellogenin by cultured hepatocytes stimulated by nonylphenol (10 μM) and estradiol (10 nM) after 4 days exposure. The results are the means of triplicate observations and the vertical bars represent the standard error. Tamoxifen significantly decreased the production of vitellogenin in both cases ($^{*}P < 0.05$, $^{***}P < 0.001$), compared to when 17β-oestradiol or 4-NP were tested alone. Tamoxifen alone significantly increased the production of vitellogenin compared with the control ($P < 0.05$).

range. Each of the xenobiotics became cytotoxic at doses between 50 and 200 μM (evidenced by dispersion of the cell aggregates and reduced viability when assessed using the Trypan blue dye exclusion test), leading to a reduction in the amount of vitellogenin secreted. In general, the cytotoxicity of the APnEOs (NP2EO, NP9EO and NP40EO), was less than that of the APs (4-tBP, 4-tOP, and 4-NP).

All of the xenobiotics were tested in the bioassay more than once (2–6-times). ED50s were calculated using each of the dose–response curves and averaged over several experiments for each compound. Table 2 shows for each compound the mean ED50, standard error, and the number of times the compound was tested. An average figure for the relative potency when compared to oestradiol is also given. The most potent compound was 4-tBP, which had a relative potency of 0.00016.

Figure 4 illustrates how the secretion of vitellogenin can be reduced by inclusion of Tamoxifen in the medium. Tamoxifen acts as an oestrogen antagonist which inhibits the binding of oestradiol to the oestrogen receptor by blocking the receptor binding sites which would normally have to be occupied by the hormone in order to produce a response; in this case, vitellogenin synthesis and secretion. This experiment was carried out to determine whether the alkylphenols and related compounds were indeed acting via the oestradiol receptor in a similar manner to oestradiol itself. The results show that 1 μM Tamoxifen itself acted as a weak agonist, causing a small but significant increase in the secretion of vitellogenin ($P < 0.05$). However, when administered simultaneously with 10 nM oestradiol or 10 μM 4-NP, the presence of Tamoxifen caused a significant reduction in the amount of vitellogenin produced.

DISCUSSION

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DISCUSSION

There have been numerous reports on the biodegradation of detergents and surfactants during sewage treatment (Giger et al., 1984, 1987). Although most of these reports have concentrated on the nonylphenol-polyethoxylates, octylphenol-polyethoxylates represent 15–20% of the total (Naylor et al., 1992). During sewage treatment, 90% of the polyethoxylate compounds (APnEO, where n = 9–40) are eliminated. As the ethoxylate chain becomes shorter, the products become more resistant to biodegradation and more lipophilic. NP1EO and NP2EO have been reported in effluent and in river water in both America and Europe (Ahel and Giger, 1985; Giger et al., 1987; Garrison and Hill, 1972; Marcomini et al., 1990; Stephanou and Giger, 1982; Thoumelin, 1991; Wahlberg et al., 1990). Because of its hydrophobicity, most of the 4-NP produced during sewage treatment is found in the sludge (Giger et al., 1984), although it has still been reported at concentrations in excess of 10 μM in the effluent (Garrison and Hill, 1972). NP1EC and NP2EC, whilst not found in raw sewage, are present in effluent at concentrations approaching 1 μM (Thoumelin, 1991; Giger et al., 1987).

In this study, we have demonstrated that many of these biodegradation products of detergents are oestrogenic to fish cells when tested at micromolar concentrations. This oestrogenic effect has previously been demonstrated in mammalian cell culture (Soto et al., 1991), although these authors limited their studies to testing the alkylphenols only. The oestrogenic activity of these compounds appears to be associated with extension of the alkyl side chain in the *para* position. When this group is moved *ortho* or *meta* to the phenolic group (as in 2-tBP or 3-tBP), activity is lost. Previous workers have also demonstrated this feature (Soto et al., 1991; Mueller and Kim, 1978). Although these compounds are weak oestrogens, this does not mean that they are not biologically important. Soto et al. (1992) demonstrated that the alkylphenols are 3-times more potent than the weakly oestrogenic pesticides o'p'DDT and kepone. We have made similar observations in our own studies (results not shown). The action of DDT in particular has been extensively studied and there is histological and anatomical evidence that at concentrations of only 2 to 5 μg/g in ovo, o'p'DDT can induce feminisation in the embryos of male birds (Fry and Toone, 1981; Fry et al., 1987).

The potency of the polyethoxylate compounds (NP2EO, NP9EO and NP40EO) appears to decrease with increasing length of the ethoxy chain. These compounds are more water soluble than the alkylphenols themselves and consequently more bioavailable to aquatic organisms. It was noted that after only one 48 h incubation, NP9EO, NP2EO and NP1EC did not appear to be as potent as 4-NP itself, whilst after 96 h, NP9EO was equally as potent, and NP2EO and NP1EC were more potent, than 4-NP itself (results not shown). The increase in the potencies of these compounds with time could be due to metabolic transformation within the hepatocytes. As with other pharmacologically active compounds, any structural alterations of the molecule caused by its metabolism will most likely affect its biological activity. Thus, com-

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pounds of weak activity may acquire greater efficacy by metabolism (Ousterhout et al., 1991; James, 1986), although in other instances compounds may be detoxified.

Because of their lipophilicity and persistence, alkylphenols are bioaccumulable. McLeese (1981) found bioconcentration factors (BCF) for nonylphenol of 280 in salmon and 10 in the common mussel. Later studies report BCFs in sticklebacks and mussels 5- and 340-times higher, respectively, than those obtained previously (Ekelund et al., 1990). Thus, the BCF found in sticklebacks was 1400. Because of the bioaccumulative properties of the alkylphenols, it is likely that they will be potent at much lower concentrations in vivo than found here, in vitro. Shiraishi et al (1989) found 40 μg per gramme of *p-tert*-pentylphenol in the adipose tissue of carp exposed to effluent from a chemical processing plant. This alkylphenol can also interact with the oestrogen receptor (Mueller and Kim, 1978). Because fish use lipid rather than carbohydrates as an energy source (Babin and Vernier, 1989), it is possible that alkylphenols stored within adipose tissue may be mobilised and transported around the body in the blood when the fat is utilized.

We have demonstrated that many of the products of the biodegradation of detergents, known to be present in sewage effluent and therefore also in rivers, are oestrogenic to fish cells. However, the consequences of the exposure of fish to these oestrogenic compounds are not yet known.

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ATTACHMENT #11

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ESTROGENIC EFFECTS OF EFFLUENTS FROM SEWAGE TREATMENT WORKS

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The occurrence of hermaphrodite fish in the lagoons of sewage treatment works led us to hypothesize that sewage effluent might contain a substance, or substances, estrogenic to fish. To test this hypothesis, we placed cages containing rainbow trout in the effluent from sewage-treatment works, and one to three weeks later measured the vitellogenin concentration in the plasma of the fish. Vitellogenin is a protein synthesized by the liver of oviparous fish in response to estradiol stimulation; it is then conveyed by the blood to the ovary, where it is sequestered by oocytes to form the yolk. Thus, the presence of vitellogenin in the plasma is indicative of estrogenic stimulation of the liver. An initial study, at a sewage-treatment works, showed that plasma vitellogenin concentrations rose rapidly and very markedly (over 1000-fold in three weeks) when trout were maintained in the effluent. An extensive nationwide survey was then conducted. Results were obtained from fifteen sewage-treatment works distributed throughout England. In all cases, exposure of trout to effluent resulted in a very pronounced increase (500 to 100,000-fold, depending on site) in the plasma vitellogenin concentration. Induction of vitellogenesis was also observed in carp, but to a much lesser extent than in trout.

The identity of the estrogenic substance is unknown. It is suggested that the two most likely possibilities are ethynylestradiol, originating from pharmaceutical use, or alkylphenol-ethoxylates (APE), originating from the biodegradation of surfactants and detergents during sewage treatment.

Laboratory studies on the potency of ethynylestradiol demonstrated that levels as low as 1 to 10 ng l^{-1} could generate the response shown by the caged fish and that positive responses may arise at 0.1 to 0.5 ng l^{-1}. Further work is in progress on the potency of APE.

KEY WORDS: sewage effluent, vitellogenesis, estrogenesis, ethynylestradiol, alkylphenol-ethoxylates

INTRODUCTION

Some of the more significant cases of environmental pollution have arisen from the purposeful use of biologically active materials. The classical example is the mid-century impact of organochlorine pesticides such as DDT; a more recent one is the effect on oysters and other molluscs of tributyltin, as used in ship paints to prevent settlement of sessile organisms (Waldock, 1986). An understanding of the biological consequences of such contamination has an *a priori* element which is not always so obvious for pollution which arises from disposal as opposed to use. For example, the adverse biological effects of PCBs and some heavy metals are of concern to us but their modes of action remain conjectural.

Following the widespread adoption of hormonal contraception procedures, general concern has been expressed in the popular media about the potential effects of constituents of contraceptive pills entering waste waters and rivers. Scientific concern has also been expressed, particularly on the possible consequences of contamination by estrogens (Richardson and Bowron, 1985; Aherne *et al.*, 1985).

The present study of estrogenic effects on fish of effluent water from sewage treatment works (STW) began as a consequence of the casual observation by anglers of hermaphrodite fish in STW lagoons and its subsequent confirmation by a follow-up survey. An attempt to assay the effluent by *in vivo* and *in vitro* mammalian methods was inconclusive and virtually uninterpretable, but a more direct and specific fish assay was available. This was based on the measurement of plasma concentrations of vitellogenin (Sumpter, 1985), the yolk precursor which is found in all egg laying animals. Vitellogenin is produced naturally by the liver in females under the control of estrogens secreted by the ovary but it can also be synthesized in both females and males following exposure of fish to exogenous estrogens (Clemens, 1978).

A vitellogenin radioimmunoassay (RIA) was developed for the rainbow trout (*Oncorhynchus mykiss*) and the overall plan for the assessment of STW effluents was to place trout in cages in or near to effluent discharges. To test the hypothesis that the constituents of the contraceptive pill contributed to the estrogenic effect of STW effluents, their potency was assessed under laboratory conditions. In addition, since many of the rivers receiving STW effluents support coarse fish rather than trout, an RIA for carp (*Cyprinus carpio*) vitellogenin was developed (Tyler and Sumpter, 1990) and used in preliminary field and laboratory tests.

MATERIALS AND METHODS

Rainbow trout were purchased from several sources. Ideally they were all-male stock produced by masculinization (Purdom, 1984). However, when it was necessary to buy ostensibly mixed-sex stock there were occasions when all the fish were females due to the adoption of all-female techniques (Bye and Lincoln, 1986) by many UK hatcheries. Fish were immature and had to be killed in order to identify their gender. Immature carp were purchased from a single supplier. All fish were acclimatised (for a few days at least) in tanks provided with mains water, predominantly from a bore-hole source.

In field trials the fish were held in galvanised steel cages and were not fed during the periods under test. Blood was sampled by heparinized syringe from the caudal sinus after anaesthesia with 2-phenoxyethanol. Fish were then killed, measured for length and sexed. Blood was centrifuged and the plasma stored in liquid nitrogen prior to assay. The vitellogenin assay was conducted as described by Sumpter (1985) or Tyler and Sumpter (1990) and was performed blind on coded samples.

Five series of field trials were performed as follows:

i. Trout were held at the main STW at which hermaphrodite fish had been observed, at a nearby but smaller STW with a largely rural catchment zone, at a MAFF Experimental Trout Culture Unit just below a small STW, and at two control sites comprising tap water supply (largely bore-hole in origin) and spring supply (the farm of origin of the fish). This was a preliminary trial and fish were exposed for varying times.

ii. Following positive results from (i), in a further preliminary trial trout were placed in effluent at the main STW for assessment of the time-dependence of the vitellogenic response.
iii. The main trial embraced the study of 30 STWs chosen throughout England and Wales to include several from each Water Authority region; the choices were made on the advice of individual Water Authorities and on separate assessments of the likelihood that trout could survive in the effluent streams.
iv. A series of trials was made using carp at selected sites used for (iii).
v. A series of trials of linked STWs, potable water intake points and intermediate sites was made using trout.

In laboratory tests of the potency of contraceptive pill constituents, trout and carp were held in 1600 l glass aquaria with a flow-through of water into which stock solutions of steroids in distilled water were fed via a peristaltic pump and mixing chamber. In one trial, treatment was *via* intra muscular injection.

The purpose of the field trials was to investigate the occurrence of estrogenic substances in STW effluents and not to quantify the vitellogenic response in relation to content and concentration of substances in the effluent. The extreme variability of effluent composition, flow rate and dilution during the trials made inter-site comparisons invalid and repeat trials under identical conditions impossible. In the data from these trials the individual levels of vitellogenin varied widely. However, the effect at the main STW and results from subsequent studies of other STW effluents were so obvious that tests of statistical significance were inappropriate, but in cases where the picture was less clear, non-parametric tests were applied to aid interpretation of the data (Conover, 1980).

Analysis of the data from the laboratory tests was with the IBM SPSS statistical package. In experiments in which all the fish in a group (usually the controls) had plasma vitellogenin levels below the detection limits of the RIA, the analysis was done on the counts per minute values obtained from the gamma counter in order to introduce variation within the group.

RESULTS

Field trial i.: Winter 1986–1987

The results shown in Table I clearly indicate that enhanced levels of plasma vitellogenin arose in male and female fish held at site A, the main STW, with some indication also of increased levels at the smaller STW (site B) and the Experimental Trout Unit. The levels in males at the control sites were at the limit of the resolving power of the assay. This was therefore the first clear indication of an estrogenic activity in STW effluent.

Field trial ii.: Spring 1988

Male fish were used and exposed to effluent at the main STW for 3 weeks or held as controls in tap water. Ten fish were sampled at the end of weeks 1 to 3 in each group. The results showed a steady climb in mean vitellogenin levels at 33 $\mu g\ ml^{-1}$ for week 1, 192 $\mu g\ ml^{-1}$ for week 2 and 373 $\mu g\ ml^{-1}$ for week 3. Variations were again large and the population standard deviations were 25, 149 and 272, respectively.

Table I Preliminary trials measuring serum vitellogenin levels in rainbow trout at specific sites.

Site	Exposure (days)	Males			Females		
			vitellogenin $\mu g\ ml^{-1}$			vitellogenin $\mu g\ ml^{-1}$	
		n	mean vitellogenin	range	n	mean vitellogenin	range
STW site A*	33	4	20.2	0.1–78.0	14	63.6	1.5–130.0
	67	9	174.2	27.0–400.0	9	407.3	87.0–880.0
STW site B	33	10	0.6	<0.02–4.7	10	1.8	0.1–8.8
Experimental trout	34	10	10.0	0.03–36.5	6	12.5	0.3–42.5
unit below STW	61	13	0.3	<0.02–1.2	10	3.5	0.3–10.0
	91	12	13.5	0.06–71.0	12	20.2	2.7–148.0
Control site	91	14	0.03	<0.02–0.05	17	1.1	0.2–2.5
mains water	Resident	7	0.04	<0.02–0.10	5	2.7	0.7–6.1
Control Farm-spring water	Resident	40	0.03	<0.02–0.15	42	1.3	0.05–6.5

*STW sites are coded to fulfil confidentiality requirements.

At the control site, levels were again around the limits of detection at 0.1, 0.06 and 0.04 $\mu g\ ml^{-1}$, respectively. Three-week periods were adopted for future STW exposures.

Field trial iii.: Summer 1988

This was the main field trial and the results are given in Table II. Despite careful choice, at 12 of the sites the fish were unable to survive and at three others works plant malfunction was judged to be responsible for the death of all fish. At each of the 15 sites where fish survived the 3-week exposure, clear evidence of large increases in plasma vitellogenin was obtained. Even in male fish, where vitellogenin is usually not detectable, the observed levels were in $mg\ ml^{-1}$ concentrations, falling within the range normally observed in mature females during egg formation. At the control sites, the levels of vitellogenin were generally low except for a very mildly elevated value at site 3. Surviving fish at most sites were in poor condition. As a check for stress-related effects, fish were placed in the influent and effluent channels of a major trout farm as poor water quality of an effluent can stress rainbow trout. No vitellogenin elevation was observed when influent fish (18 females: mean vitellogenin 32.6 $\mu g\ ml^{-1}$)were compared to those in the effluent (13 females, 10.5 $\mu g\ ml^{-1}$).

Field trial iv.: Winter 1988

Immature common carp (*Cyprinus carpio*) were deployed at ten of the STWs previously used successfully for trout. At one, site K, plasma samples were taken weekly for 6 weeks. At the others, samples were taken after three-week exposures. The results are given in Table III. Vitellogenin levels were very low overall compared to those for rainbow trout and amongst the controls a few fish gave surprisingly high values. A non parametric test of significance Kolmogorov-Smirnov (Conover, 1980) indicated that significantly high values were obtained at sites J, L and N and the series at site K did seem to show an increasing trend albeit not of statistical

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12.5	0.3–42.5
3.5	0.3–10.0
20.2	2.7–148.0
1.1	0.2–2.5
2.7	0.7–6.1
1.3	0.05–6.5

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Table II Results of nationwide survey of estrogenic activity in STW effluents. Means of samples of 8 to 20 surviving fish.

	Site	Vitellogenin levels in μg ml⁻¹ following 3 weeks exposure	
		Male	Female
Control sites	1	–	4.5
(Spring or tap water)	2	0.05	–
	3	1.80	88.3
	4	0.05	23.5
	5	0.22	–
STW sites	A	–	470
(final effluent)	C	23	–
	D	3 100	–
	E	147 000	112 000
	F	–	10 000
	G	–	48 000
	H	7 600	6 000
	I	2 100	3 800
	J	–	13 200
	K	–	10 500
	L	–	3 000
	M	54 000	–
	N	65 000	–
	O	19 200	–
	P	15 600	–
	12 sites	Fish unable to survive	
	3 sites	Works failure	
	1 site	Cage and fish stolen	

Table III Plasma vitellogenin (μg ml⁻¹) levels in common carp after 3 weeks exposure at STW sites. At site K fish were maintained for 6 weeks and bled weekly.

STW site	n	mean	sd	max	min
K week 1	12	0.02	0.02	0.07	0.01
" 2	12	0.04	0.03	0.09	0.01
" 3	20	0.06	0.03	0.11	0.03
" 4	12	0.10	0.00	0.10	0.10
" 5	12	0.20	0.53	1.85	0.20
" 6	12	0.34	0.86	3.00	0.01
E	20	0.64	2.56	11.50	0.01
Q	20	0.17	0.25	1.00	0.01
R	20	1.05	4.46	20.00	0.01
L	20	2.16*	2.50	8.50	0.02
S	19	1.20	2.32	9.50	0.01
A	20	0.43	1.88	8.40	0.01
J	20	15.64*	5.67	20.00	3.15
C	20	0.09	0.09	0.30	0.01
N	20	2.28*	1.96	5.80	0.01
Controls	40	0.55	2.23	13.00	0.002

*Significantly higher than control levels.

significance with the test used. These conclusions must be treated with caution. Due to a delay in the placement of fish and an early onset of cold weather, the water temperatures on sites were around 5°C. It is likely that this is too low for normal vitellogenesis in carp, a species for which a normal English summer is too cold for natural spawning. (Hernandez *et al.*, 1992).

Field trial v.: Summer 1989

This trial was conducted because of concern for public health raised by the earlier work, even though tap water controls had consistently given negative results and a very early assay of rainbow trout held as 'sentinels' in potable water intake protection tanks by Water Authorities had also proved negative. Forty two females and 14 males were sampled from 8 localities and the mean vitellogenin levels found were 0.83 and 0.11 $\mu g\ ml^{-1}$, respectively.

Four rivers were chosen where fish in cages could be deployed at a STW, a downstream abstraction point and at an intermediate position. This requirement meant that previously used STWs were not appropriate. The results are shown in Table IV. Technical problems rendered this trial incomplete, despite an attempt to replicate it. Enhanced vitellogenin levels were observed in male trout at the STWs

Table IV Vitellogenin levels ($\mu g\ ml^{-1}$) in male rainbow trout at STW, abstraction points downstream and intermediate localities. Means and SEM of ten fish per sample.

River	Month	Control	STW	Intermediate	Abstraction
V	May	<0.005	898 ± 233**	0.016 ± 0.01 NS	0.076 ± 0.05*
	August	<0.010	2,154 ± 395**	NO TEST	<0.010 NS
W	May	<0.005	x	0.290 ± 0.21*	0.240 ± 0.16*
	August	<0.010	x	0.110 ± 0.05*	+
X	May	<0.010	2,078 ± 614**	NO TEST	<0.010 NS
T	May	<.005	+	<0.005 NS	<0.005 NS

x Placement of fish precluded by technical detail in Home Office licence
\+ Fish died
*P<0.05)
**P<0.001) Statistical significance of response compared to controls.
NS – Not Significant)

but little effect was evident downstream for rivers V and X. For river W there was evidence for a slight increase in vitellogenin levels in downstream abstraction and intermediate points. This is a slow, lowland river whereas river V is a fast-flowing chalk stream. The results suggest that substantial dilution in rivers with strong flows may largely dissipate the estrogenic effect, but this may not be the case in slow flowing rivers. On the fourth river T, fish died at the STW but showed no evidence of enhanced vitellogenin levels at either of the downstream localities.

LABORATORY TRIALS

An *a priori* argument at the outset was that constituents of the contraceptive pill might be implicated in an estrogenic effect of waste waters. The principal estrogen